

THE ROLE OF DESMOSOMAL CADHERINS IN COLORECTAL TUMOURIGENESIS

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A thesis submitted to the
University of Birmingham
For the degree of
DOCTOR OF MEDICINE

School of Cancer Sciences
University of Birmingham
May 2012

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ABSTRACT

In cancer, loss of intercellular contact contributes to tumour progression and invasion. Desmosomal cadherins are essential constituents of desmosomes – intercellular junctions that confer significant adhesive strength to epithelial tissues and cardiac muscle. Although changes in desmosomal components have been noted in a variety of cancers previously, this investigation has shown for the first time altered desmocollin expression in colorectal cancer.

Real-time PCR and western blotting were used to assess desmocollin expression in a series of colorectal cancer and matched normal tissue samples. Loss of desmocollin 2 expression was observed in the cancer samples. In addition, de novo expression of desmocollins 1 and 3, which are not normally expressed in the colon, was observed. Desmoglein gene expression was also altered in the cancer samples. Although classical cadherin switching is a hallmark of the epithelial-mesenchymal transition, desmocollin switching has not previously been reported.

Further experiments, to investigate the effect of loss of desmocollin 2 and desmoglein 2 on the behaviour of cultured cells were performed. In addition, experiments were carried out to identify those transcription factors that regulate desmosomal cadherin gene expression in the colon. Transcription factors of the CCAAT/enhancer-binding proteins family act as transcriptional activators of desmosomal cadherin promoters in colonic cells.

For Guy, Eve and Aiden,

Despite not knowing anything about desmosomes, you knew enough to know this work matters to me.

I could not have done it without you.

ACKNOWLEDGEMENTS

I would like to thank my supervisors, Dr Martyn Chidgey and Professor Dion Morton all the help, patience and support, throughout the duration of this work.

I am also extremely grateful to my colleagues, Denise Youngs, Dr Carolyn Jones and Dr Germaine Caldwell, for all their advice and assistance, as well as for steering me in the right direction in the lab.

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CHAPTER 1

INTRODUCTION

1.1 Colorectal Cancer

1.1.1 Epidemiology

Colorectal cancer (CRC) is a major cause of mortality and morbidity in the United Kingdom and other parts of the developed world (reviewed by Parkin, Bray et al. 2005). It is the third most common cause of cancer death in the UK after lung and prostate cancer in men and after lung and breast cancer in women (Westlake and Cooper 2008). Over 30,000 new cases were diagnosed in England in 2006, with more than 90% of these being in the over 55 years age-group (Cancer Statistics Registrations 2006). Whilst the incidence of colorectal cancer has remained largely stable over the past decade, mortality rates continue to fall due to improved diagnosis and treatment. However, with the implementation of the National Health Service Bowel Cancer Screening Programme, it is likely that initially the incidence of colorectal cancer will increase, but deaths from colorectal cancer will be avoided as it will be diagnosed at an earlier stage.

Colorectal cancer incidence and mortality are both higher in men than women. Age-standardised rates of colorectal cancer are higher in males (19.1 per 100,000) than females (14.4 per 100,000) globally (Boyle and Leon 2002).

Incidence of CRC is highest in the United States of America, Canada, Japan and New Zealand (Parkin, Bray et al. 2005) and migration studies have shown the environmental factors result in migrants acquiring similar risk to the local population (McCredie, Williams et al. 1999).

1.1.2 Aetiology

Although the exact cause of colorectal cancer is not yet fully understood, both environmental and genetic factors play significant roles in the development of these tumours.

1.1.2.1 Acquired Risk Factors for Colorectal Cancer

Diet may influence the development of colorectal neoplasia through acting directly at the gut mucosa-lumen interface, or indirectly by changing the metabolism of key nutrients, hormones and growth factors throughout the body. A number of different foodstuffs have been implicated in the development of colorectal neoplasia but overall the diet of the westernised world seems to be instrumental (Dixon, Balder et al. 2004). The Second Report of the World Cancer Research Fund concluded that high red meat and processed meat intake was convincingly implicated in colorectal tumourigenesis, whereas dietary fibre, garlic, milk and calcium probably protect against CRC and fruit and vegetables, folate-rich foods, vitamin D or selenium may protect against CRC (World Cancer Research Fund / American Institute for Cancer Research 2007). Furthermore, a recent study has shown

recurrence and survival rates to be significantly worse in colorectal cancer patients with less fibre, more fat and more sugar in their diets (Meyerhardt, Niedzwiecki et al. 2007). Murine models have demonstrated that when fed a westernised diet high in fat, but low in fibre, folate, calcium, and vitamin D, tumours could be induced in normal *C57B1/6* mice without carcinogen exposure (Newmark, Yang et al. 2001). In addition, colonic neoplasia can be prevented by elevating dietary calcium and vitamin D in the westernised diet (Yang, Kurihara et al. 2008).

The strongest evidence exists in relation to high levels of red and processed meat consumption. One large meta-analysis, including 26 studies involving a total of over 15,000 subjects, demonstrated a 20% increased risk of developing colorectal cancer in the group with the highest consumption when compared to that with the lowest (Huxley, Ansary-Moghaddam et al. 2009). In addition, a high beef diet induced significantly more tumours in *Apc^{Min}* (Multiple intestinal neoplasia) mice (Mutanen, Pajari et al. 2000) and has been implicated in tumour formation in rodents and humans (Ferrucci, Sinha et al. 2009). Various mechanisms for the tumourigenic role of meat consumption have been proposed, including the formation of meat mutagens, such as heterocyclic amines, which are generated by cooking meat at high temperatures (Sinha, Rothman et al. 1998) and the alteration of protein kinase C (PKC) levels in rat colonic mucosa (Pajari, Oikarinen et al. 2000).

Fruit and vegetable intake was previously thought to have a protective role against colorectal cancer. The Nurses' Health Study showed there was no association between colon cancer and fruit and vegetable consumption (Willett, Stampfer et al. 1990). The role of fibre in colorectal tumourigenesis has been extensively investigated. Several meta-analyses have been performed on data from case-control studies. Young et al have summarised these and concluded that there is a consistent protective effect from fibre and consumption of 30g per day reduces the risk of developing colorectal cancer by 50% (Young, Hu et al. 2005). Prospective colorectal cancer and polyp prevention trials have failed to demonstrate the benefit of fibre as a primary prevention tool but such studies have been limited by short follow up periods (Asano and McLeod 2002). Initially it was proposed that fibre provided stool bulk and volume and decreased transit time, reducing the exposure time of the colonic mucosa to carcinogens (Burkitt 1971). Other mechanisms involving fermentation (increased production of short chain fatty acids, reduced solubility of bile salts), prebiotic action of gut flora and whole-body metabolism (reduced insulin resistance) have now been recognised (Kim 2000). In conclusion, although the exact roles and relative impact of individual macro- and micro-nutrients on the development of colorectal cancer has yet to be fully elucidated, the Western diet confers an increased risk of developing colorectal adenomas and carcinomas.

Physical activity appears to have a protective effect against colorectal cancer (Huxley, Ansary-Moghaddam et al. 2009) with stronger evidence for its role in

colon rather than rectal cancer. Aside from weight control, the mechanisms through which activity reduces colorectal cancer risk include decreased gastrointestinal transit time, thus reducing exposure time to carcinogens, altered prostaglandin secretion and bile acid metabolism (Friedenreich and Orenstein 2002). Several studies have shown an increased risk of colorectal cancer with obesity. The association appears to be stronger for men than women and colonic rather than rectal lesions (Moghaddam, Woodward et al. 2007). The excess of obesity-related cancers seen in men appears to be due at least in part to the central distribution of fat. Women are more frequently 'pear-shaped' (gluteofemoral fat distribution) but also showed a positive association of colon cancer with increasing waist-to-hip ratio (MacInnis, English et al. 2006). Data from the Framingham cohort also concluded that waist circumference was a stronger predictor of lifetime risk of developing colon cancer than body mass index (Moore, Bradlee et al. 2004).

Physical inactivity, central adiposity and obesity all result in high levels of insulin production, as do type II diabetes mellitus and hypertriglyceridaemia. (McKeown-Eyssen 1994). Conditions associated with high levels of circulating insulin are at increased risk of colorectal tumourigenesis. (Tran, Medline et al. 1996). Insulin acts to increase levels of Insulin-like Growth Factor (IGF)-1 which inhibits apoptosis and stimulate proliferation of colon cancer cells. Acromegaly and greater adult height are also associated with raised levels of IGF-1 and are associated with increased CRC risk (Giovannucci 2001).

Although early studies investigating a relationship between cigarette smoking and colorectal cancer failed to demonstrate a link, more recent evidence from large meta-analyses suggests that in fact, smoking is associated with both adenoma (Botteri, Iodice et al. 2008) and colorectal tumour formation (Liang, Chen et al. 2009). Tobacco smoking is well known to release a range of carcinogens which reach the colonic mucosa both via the bloodstream and through digestion. A recent review of the evidence has indicated that daily cigarette consumption; number of pack-years; age of initiation and duration of smoking habit are all factors adding to the increased risk of colorectal cancer in smokers, which is thought to contribute in up to 20% of cancers (Chan and Giovannucci 2010). Cigarette smoking appears to be more strongly associated with microsatellite instability in colorectal cancer, in particular with CIMP-positive (CpG Island Methylator Phenotype) tumours with *BRAF* mutations – a picture observed with the sessile serrated adenoma pathway (Slattery, Curtin et al. 2000; Samowitz, Albertsen et al. 2006).

1.1.2.2 Co-Morbid Conditions

The association between inflammatory bowel disease (IBD) and CRC is well recognised, having been first described by Crohn and Rosenberg in 1925 (Crohn and Rosenberg 1925). Colorectal cancer arising against a background of either Crohn's disease or Ulcerative Colitis represents for only 1-2% of all cases of CRC but is responsible for the death of 10-15% of patients with inflammatory bowel disease (Munkholm 2003). The affected patients are younger than those with sporadic cancers, with one meta-

analysis giving the average age at diagnosis as 43.2 years (Eaden, Abrams et al. 2001). This, and other studies have concluded that extensive disease (Ekbom, Helmick et al. 1990) and duration of more than 10 years are the most important risk factors for the development of colitis-associated cancer (Ekbom, Helmick et al. 1990), as well as primary sclerosing cholangitis, a family history of colorectal cancer and the degree of inflammation (Lakatos and Lakatos 2008).

Numerous studies have investigated the relationship between gallstones, cholecystectomy and the development of colorectal adenomas and cancer. This has been difficult to characterise as there many risk factors that are common to both, in particular, diet. A large prospective study that stratified for such confounding variables found a relative increased risk of up to 1.58 times when compared to the control group (Schernhammer, Leitzmann et al. 2003). Various hypotheses have been postulated including altered bile salt metabolism and increased faecal fat content in cholecystectomised patients (Morvay, Szentleleki et al. 1989; Zuccato, Venturi et al. 1993).

Several studies have concluded that type 2 diabetes results in an increased risk of colorectal cancer of around 30% (Larsson, Orsini et al. 2005) and also an increased risk of mortality from colorectal cancer (Jiang, Ben et al. 2011). This has been attributed to the effects of hyperinsulinaemia and insulin resistance (reviewed by Giovannucci 1995; Giovannucci 2001).

Carcinoma development following urinary diversion into the sigmoid colon is a well-recognised long term complication of ureterosigmoidostomy. Although largely historical now as this procedure has been superseded by other forms of bladder reconstruction, it is important to recognise this as a cause of colorectal neoplasia as it has a latent period of 20-30 years and an incidence of up to 2.5% (Kälble, Hofmann et al. 2011).

1.1.2.3 Inherited Colorectal Cancer Syndromes

Approximately 5% of all cases of colorectal cancer can be ascribed to syndromes that have been characterised by their genetic alteration, clinical manifestations or both (Table 1.1). However, it has been suggested that in up to 30% of all cases of colorectal cancer inherited factors may be involved, although the genetic events involved have not been fully elucidated as yet (Jasperson, Tuohy et al. 2010).

The recognised syndromes can be divided into those exhibiting multiple polyp formation and those which do not. Although the colorectal tumours that feature in Lynch syndrome (also known as Hereditary Non-Polyposis Colorectal Cancer, HNPCC) still arise in adenomas, polyposis is rare. It is the most common form of inherited colorectal cancer, accounting for up to 5% of cases (Lynch and de la Chapelle 1999; Hampel, Frankel et al. 2008), with a lifetime risk of developing a colorectal cancer of 70%, as well as other cancers (Stoffel, Mukherjee et al. 2009). Patients in this group are often younger, with

cancers and polyps that are located more proximally in the colon and are at risk of extracolonic malignancy (see Table 1.1). They exhibit high levels of microsatellite instability, as a result of germline mutations in the mismatch repair genes (*hMLH1*, *hMLH2*, *hMSH6*, *hPMS2*, *EpCAM*) (Söreide, Janssen et al. 2006; Jasperson, Tuohy et al. 2010). Clinical guidelines (Amsterdam criteria I and II) are now used in conjunction with genetic information (revised Bethesda criteria) to identify at-risk individuals who require further investigation (Umar, Boland et al. 2004).

Familial adenomatous polyposis (FAP) accounts for <1% of all colorectal cancer cases. It arises due to mutations of the *APC* (adenomatous polyposis coli) gene on chromosome 5q (Bodmer, Bailey et al. 1987; Groden, Thliveris et al. 1991) and results in the development of thousands of adenomas throughout the colon and rectum. These develop in early adolescence and the risk of developing an associated cancer is 100% if untreated. The mean age of developing colorectal cancer in these individuals is 39 years. Attenuated FAP exhibits far fewer polyps (average number is 30), with a lifetime cancer risk of 69% (Burt, Leppert et al. 2004). *MUTYH*-associated polyposis (MAP) is a genetically distinct condition with a phenotype similar to FAP. It arises due to biallelic mutations of the *MUTYH* gene on chromosome 1, resulting in the development of polyposis by the fourth decade and a lifetime risk of colorectal cancer of 100% by 60 years of age (Cleary, Cotterchio et al. 2009).

Peutz-Jeghers syndrome and juvenile polyposis syndrome both result in the development of hamartomatous polyposis with increased risk of colorectal cancer. These patients often present in adolescence or young adulthood with symptoms from extracolonic manifestations of these syndromes (listed in Table 1.1), with both conditions presenting a lifetime risk of colorectal cancer of 39%.

Table 1.1 Features of colorectal cancer syndromes

Syndrome (inheritance pattern)	Genes involved	Type of cancer	Other features
Lynch syndrome (AD)	<i>hMLH1</i> , <i>hMLH2</i> , <i>hMSH6</i> , <i>hPMS2</i> , <i>EpCAM</i>	Colon, endometrium, stomach, pancreas, small bowel, ovary, hepatobiliary, kidney, CNS	rare
Familial adenomatous polyposis (AD)	<i>APC</i>	Colon, duodenum, stomach, pancreas, thyroid, liver, CNS	Florid polyposis throughout colon and rectum, gastric and duodenal polyposis, CHRPE, epidermoid cysts, osteomas, desmoid tumours (Gardner syndrome)
Attenuated FAP (AD)	<i>APC</i>	Colon, duodenum, thyroid	<100 colonic polyps, upper GI polyps
<i>MUTYH</i> -associated polyposis (AR)	<i>MUTYH</i>	Colon, duodenum	<100 colonic polyps, duodenal polyps
Peutz-Jeghers syndrome (AD)	<i>STK11</i>	Breast, colon, pancreas, stomach, ovary, lung, small bowel, uterine, testis	Peri-oral pigmentation, GI hamartomas
Juvenile polyposis syndrome (AD)	<i>SMAD4</i> , <i>BMPR1A</i>	Colon, stomach, pancreas, small bowel	GI hamartomas, hereditary haemorrhagic telangiectasia, congenital defects
Hyperplastic polyposis		colon	Hyperplastic polyps, sessile serrated polyps

AD, autosomal-dominant; AR, autosomal-recessive; CNS, central nervous system; FAP, familial adenomatous polyposis; CHRPE, congenital hypertrophy of the retinal pigment epithelium (adapted from Jasperson, Tuohy et al. 2010)

Once features of these distinct inherited colorectal cancer syndromes have been identified, genetic testing of the affected individual and at-risk relatives can be performed. This allows screening colonoscopy and risk-reducing surgery to be performed. Surveillance schedules vary according to the syndrome but would aim to start as a teenager or young adult or at least 5 years younger than the youngest affected relative.

1.2 Models of colorectal cancer pathogenesis

1.2.1 The adenoma-carcinoma sequence

It is now widely regarded that majority of sporadic colorectal cancers arise in adenomatous polyps, which may be situated anywhere within the colon or rectum (Leslie, Carey et al. 2002). However, not all adenomas will develop into cancers and when they do, it is usually a slow process that takes many years. Although there are no reliable criteria that can be used to predict which have malignant potential, a number of features have been recognised as risk factors for malignant transformation (Table 1.2) (Hardy, Meltzer et al. 2000).

In 1990, Fearon and Vogelstein proposed a genetic model for colorectal tumourigenesis, in which the transition from normal mucosa to adenoma to carcinoma involved the stepwise accumulation of genetic events (Fearon and Vogelstein 1990). A further intermediary step was subsequently identified

when aberrant crypt foci were shown to be the precursors to the development of adenomas (Takayama, Katsuki et al. 1998) (see Fig 1.1).

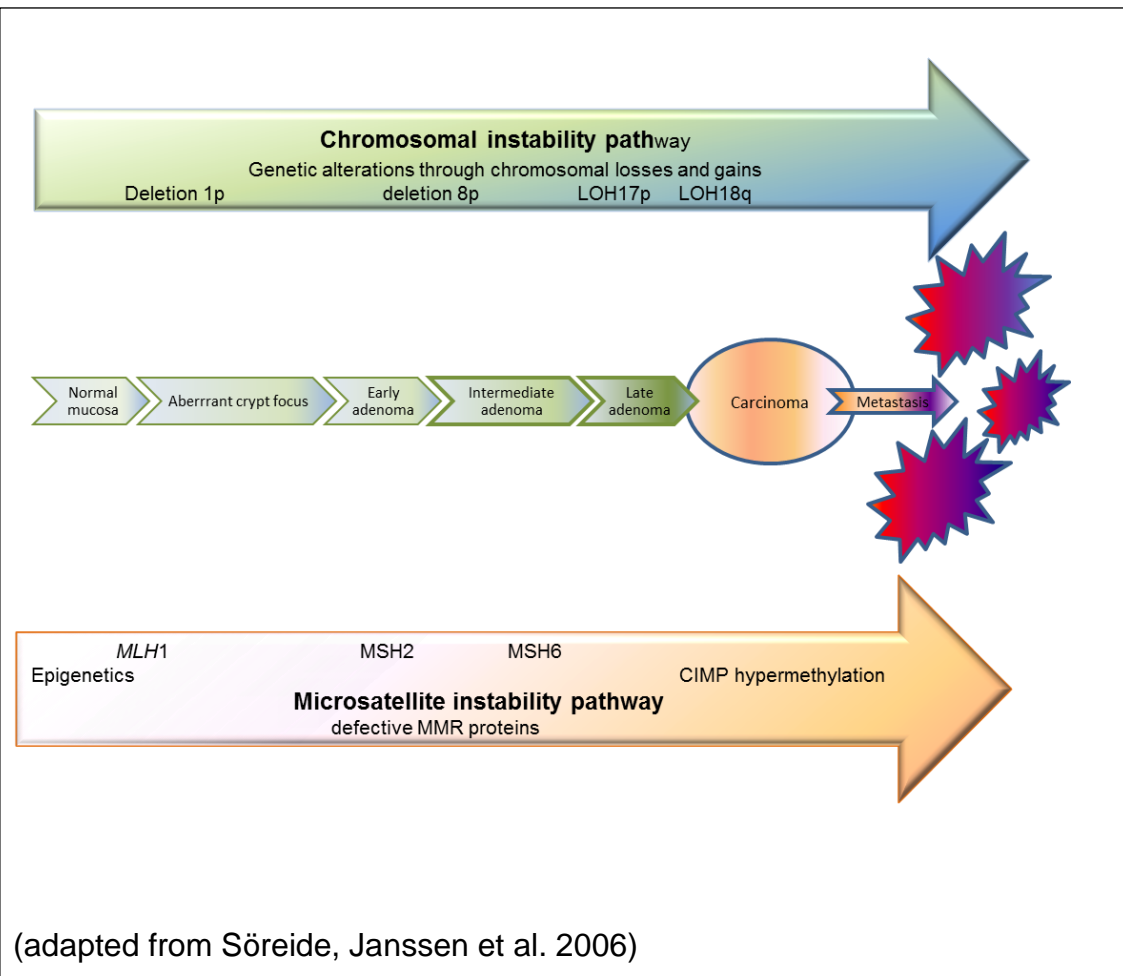
Fearon acknowledged it is the accumulation of genetic alterations rather than the order, and numerous other events have been observed and added to the model since it was originally described. Two main molecular pathways have been described: chromosomal instability and microsatellite instability. More recently it has been recognised that not all colorectal cancers arise through this traditional model, which has resulted in an additional pathway being described, the serrated pathway.

Table 1.2 Features of colonic polyps associated with malignant transformation

High Risk	Low Risk
Large size (>1.5cm)	Small size (<1cm)
Sessile or flat	Pedunculated
Severe dysplasia	Mild dysplasia
Presence of squamous metaplasia	No metaplasia
Villous architecture	Tubular architecture
Polyposis	Single polyp

(adapted from Hardy, Meltzer et al. 2000)

Fig 1.1 The adenoma-carcinoma sequence



1.2.2 Chromosomal instability

The chromosomal instability pathway is seen in about 75% of sporadic cancers and involves allelic losses (loss of heterozygosity (LOH) at 17p and 18q) and chromosomal alterations which result in tumour aneuploidy (in particular, at chromosome 5q).

Although germline mutations of the *APC* gene are responsible for the development of FAP, somatic mutations of this gene or allelic losses at 5q have been identified in 70-80% of sporadic colorectal tumours (Vogelstein, Fearon et al. 1988; Powell, Zilz et al. 1992). Mutation of the *APC* gene on chromosome 5q takes place early in the adenoma-carcinoma pathway (Powell, Zilz et al. 1992), as the majority of sporadic adenomas, as well as 5% of aberrant crypt foci have demonstrated this loss (Otori, Konishi et al. 1998). In addition, inactivation of the *APC* gene by promoter methylation has been demonstrated in 18% of colorectal cancers (Esteller, Sparks et al. 2000)

The APC protein is a large multi-functional protein with roles in many varied cellular processes (Pino and Chung 2010), but its role in the Wnt signalling pathway is key to the development of colorectal cancer (Munemitsu, Albert et al. 1995). The functional, wild-type APC protein forms a complex with Wnt components, β -catenin and GSK-3 β (Rubinfeld, Souza et al. 1993; Rubinfeld, Albert et al. 1996), allowing excess β -catenin to be broken down via ubiquitination and proteasomal degradation. However, mutant APC proteins often have impaired β -catenin binding ability (Kinzler and Vogelstein 1996), resulting in the intra-cellular accumulation of β -catenin (Munemitsu, Albert et

al. 1995). This then translocates to the nucleus, where it initiates transcriptional activation through its interaction with the TCF/LEF family of transcription factors (Mann, Gelos et al. 1999). Increased β -catenin/TCF mediated transcriptional activity has been observed not only with *APC* mutations, but also with mutations of the β -catenin gene, providing another mechanism through which this system can be activated (Morin, Sparks et al. 1997; Sparks, Morin et al. 1998).

Mutations of the *K-ras* oncogene on chromosome 12p are also thought to take place early in the development of colorectal cancer. These mutations have been observed with similar frequency in carcinomas and large adenomas, at around 30-40% (Bos, Fearon et al. 1987; Bos, Fearon et al. 1987; Forrester, Almoguera et al. 1987), but are less commonly seen in small adenomas (Scott, Bell et al. 1993). These mutations result in a constitutively active ras protein, which is involved in signal transduction of a variety of processes that regulate normal proliferation and differentiation (reviewed in Pino and Chung 2010). Furthermore, *K-ras* is a down stream effector of the epidermal growth factor receptor (EGFR) pathway. The EGFR is a tyrosine kinase receptor that is overexpressed in up to 80% of colorectal cancers and is associated with poor prognosis. Chemotherapeutic agents that inhibit EGFR overexpression are used in the treated of advanced colorectal cancer. *K-ras* mutation may be used to predict response to treatment.

The tumour suppressor gene *p53* is located on chromosome 17p and is widely regarded as the 'guardian of the genome'. It is the regulator of numerous functions including apoptosis, cell cycle control and differentiation (Lane 1992). Loss of *p53* function is thought to contribute to the transition from adenoma to carcinoma as missense mutations or 17p allelic loss are far less frequently observed in adenomas than colorectal cancers (Leslie, Carey et al. 2002).

Loss of heterozygosity at chromosome 18q is a common event in colorectal cancer, with changes identified in 70% of sporadic tumours, increasing in frequency with advanced stage (Fearon and Vogelstein 1990). This led to a search for a potential tumour suppressor gene within this region. The original candidate, *DCC* (Deleted in Colorectal Carcinoma) (Fearon, Cho et al. 1990) has subsequently been refuted in this role (Cho, Oliner et al. 1994). *SMAD2* and *SMAD4* also made attractive candidates for the role as they have been implicated in a variety of human cancers (Riggins, Kinzler et al. 1997) and act as mediators of *TGF β* -signalling (Zhou, Buckhaults et al. 1998), a mechanism also implicated in the microsatellite instability pathway of colorectal carcinogenesis (Parsons, Myeroff et al. 1995). However, mutations in these genes were seen in only 10-20% of colorectal tumours examined for this (Takagi, Koumura et al. 1998). The *Cables* gene also maps to chromosome 18q and through allelic loss and promoter methylation, exhibits reduced expression in 70% of sporadic colorectal cancers (Park do, Sakamoto et al.

2007). Although LOH at 18q is a common event in colorectal tumourigenesis, no single gene within this region has been identified as the main target.

The COX2 gene on chromosome 1q is overexpressed in 40% of adenomas and 85% of cancers (Eberhart, Coffey et al. 1994). In addition, one study found that COX2 expression correlated with increasing dysplasia in adenomas, underlying its importance in the adenoma-carcinoma sequence (Sato, Yoshinaga et al. 2003). It mediates its tumourigenic effects via prostaglandin E2 in response to a variety of growth factors and inflammatory mediators. Blockade of this pathway has shown to reduce adenoma formation in an APC knockout mouse model (Oshima, Murai et al. 2001), as well as reduce adenoma recurrence in clinical trials (Arber, Eagle et al. 2006), which generated great enthusiasm for these agents in chemoprevention. Despite a wealth of evidence to support the role of aspirin and COX2 inhibitors in colorectal cancer prevention, concern about side effects has held back their use in the general public at present.

1.2.3 Microsatellite Instability

Microsatellites are short repetitive dinucleotide sequences that occur throughout the genome. Microsatellite instability (MSI) arises due to defects in DNA mismatch repair genes (eg *hMLH1*, *hMSH2*, *hMSH6*, *hPMS2*). These genes normally correct errors that accumulate during DNA replication, however, persistence of these replication errors results in frameshift mutations and base-pair substitutions. This mechanism is thought to be responsible for the inherited colorectal cancers seen in Lynch syndrome patients, with 90% of

patients having a germline mutations in *hMLH1* and *hMSH2* (Söreide, Janssen et al. 2006). Microsatellite instability has also been detected in 12-28% of sporadic colorectal cancers (Ionov, Peinado et al. 1993; Thibodeau, Bren et al. 1993) but takes place via a different mechanism – promoter silencing by methylation (Kane, Loda et al. 1997). The CpG Island Methylator Phenotype (CIMP) has been observed in up to 50% of sporadic colorectal cancer (Toyota, Ahuja et al. 1999). Methylation is also associated with increasing age and as a response to inflammation (reviewed by Boland and Goel 2010).

The errors that persist as a consequence of defective mismatch repair result in the accumulation of mutations of other genes known to play a role in colorectal cancer. However, genes other than the DNA mismatch repair genes, that also exhibit microsatellite instability, have been implicated in the development of this form of colorectal cancer, eg transforming-growth factor β -type II receptor and *BAX*. Alterations in the transforming-growth factor β -type II receptor have been demonstrated in 90% of Lynch syndrome tumours (Parsons, Myeroff et al. 1995), which acts as a tumour suppressor in the colonic epithelium (Markowitz, Wang et al. 1995). *BAX* gene expression is altered in 35% of tumours with microsatellite instability, resulting in defective apoptosis mechanisms (Trojan, Brieger et al. 2004; Fernandez-Peralta, Nejda et al. 2005).

The molecular differences between the chromosomal instability pathway and the microsatellite instability pathway manifest themselves at both a clinical and pathological level. Microsatellite instability is more common in female patients, as well as in tumours in the proximal colon. Both synchronous and metachronous tumours are more common in microsatellite unstable patients. Despite displaying features associated with poor prognosis (Wright, Dent et al. 2000), these patients appear to have improved survival compared to chromosomal instability tumours (Gryfe, Kim et al. 2000; Popat, Hubner et al. 2005).

1.2.4 Serrated pathway of colorectal tumourigenesis

Although the majority of colorectal cancers arise in adenomas, an alternative mechanism has been identified – the serrated pathway, which is thought to be involved in the development of up to 20% of sporadic cancers (Bauer and Papaconstantinou 2008). The precursor lesions, which include traditional serrated adenoma (TSA), mixed polyp and sessile serrated adenoma (SSA), are morphologically similar to a hyperplastic polyp, but have malignant potential. The molecular pathways and the natural history of these polyps have not been fully elucidated, but has been shown to involve oncogenic activation of mitogen-activated protein kinase (MAPK) signalling pathway. Sessile serrated adenomas also exhibit microsatellite instability and CpG-island methylator phenotype (Chan, Zhao et al. 2003), along with *BRAF* mutations (Davies, Bignell et al. 2002). They seem to adopt a similar clinical picture to other tumours exhibiting microsatellite instability, in that they are

more commonly observed in middle-aged women with right-sided tumours. The addition of *BRAF* mutation does not seem to affect the prognostic advantage of microsatellite unstable tumours (Samowitz, Sweeney et al. 2005). The traditional serrated adenoma accounts for <1% of all colorectal polyps.

1.3 Role of cell adhesion in the adenoma-carcinoma sequence

Stable intercellular adhesion is essential for tissue integrity, both during development and post-embryonic life. In cancer, loss of cell-cell contacts allows tumours to progress, invade surrounding tissues and eventually metastasise. Cell adhesion can be mediated by various junctional complexes, including adherens junctions (AJ), desmosomes, gap junctions and tight junctions. Adherens junctions and desmosomes share both structural and functional similarities, although these have been more extensively studied in adherens junctions. Alterations in AJ components are well recognised in several diseases, including cancer. Loss of E-cadherin function due to germline mutations results in hereditary diffuse gastric cancer demonstrating its role as a true tumour suppressor gene (Guilford, Hopkins et al. 1998). Loss of function has been demonstrated in many other cancers, including breast, prostate, colon, kidney and skin (Semb and Christofori 1998). Loss of E-cadherin is a late event in the adenoma-carcinoma sequence contributing to the tumour's ability to invade and metastasise (Tsanou, Peschos et al. 2008). Another key component of AJs involved in cancer is β -catenin. β -catenin acts as a transcriptional activator in the wnt signal transduction pathway and

failure to degrade β -catenin, as a result of mutations in the tumour suppressor adenomatous polyposis coli (*APC*), is associated with tumours in a number of different tissues.

Like AJs, desmosomes also contain cadherins and armadillo proteins as well as other proteins, including desmoplakin. Although no mutations in desmosomal components have been identified thus far it has been proposed that desmosomes could also have a role in the development of cancer.

1.4 Desmosomes

Desmosomes are intercellular junctions that mediate adhesion by linking the intermediate filament networks of adjacent cells. They are found in all epithelial tissues, as well as cardiac muscle and the follicular dendritic cells of lymph nodes (Schmidt, Heid et al. 1994). Desmosomes confer strong cell-cell adhesion and are particularly abundant in tissues that experience mechanical stress, e.g. stratified epithelia and cardiac muscle. Desmosomal composition not only varies between different tissues but also within the same cell type (Getsios, Huen et al. 2004).

1.4.1 Desmosome structure

At an ultrastructural level, desmosomes consist of an electron dense cytoplasmic plaque at either side of a central core, which occupies the gap between adjacent cells (Green and Gaudry 2000). The cytoplasmic region is

further divided into the outer and inner dense plaque, which provides the location for the insertion of intermediate filaments. Desmosomes confer structural continuity on entire tissues by linking the intermediate filament networks of all the cells in the tissue (Kowalczyk, Bornslaeger et al. 1999). Desmosomes are complex, multimolecular structures that are made up of proteins that belong to one of three families: the desmosomal cadherins, the armadillo proteins and the plakins (Chidgey 2011). The individual components may vary according to cell type, differentiation status and disease state.

1.4.2 Desmosomal cadherins

There are 2 types of desmosomal cadherin, desmocollin and desmoglein (Fig.1.2). Seven desmosomal cadherins has been identified in humans so far. There are three desmocollins (*DSC1-3*) (Parker, Wheeler et al. 1991) (Kawamura, Watanabe et al. 1994), and four desmogleins (*DSG1-4*) (Koch, Walsh et al. 1990; Koch, Goldschmidt et al. 1991; Wheeler, Buxton et al. 1991; Wheeler, Parker et al. 1991; Schafer, Koch et al. 1994; Kljuic, Bazzi et al. 2003). Two further desmocollins have been identified in mice (*Dsc5-6*). Each desmosomal cadherin isoform is encoded by a distinct gene (Buxton, Cowin et al. 1993; King, Arnemann et al. 1993; Buxton, Wheeler et al. 1994) and these are clustered together at chromosome 18q12.1 (Hunt, Sahota et al. 1999).

The individual desmosomal cadherin isoforms are expressed in a cell-type and differentiation specific manner (Buxton, Cowin et al. 1993; Koch and Franke 1994) (Garrod, Chidgey et al. 1996). DSC2 and DSG2 are ubiquitous in all desmosome forming tissues, and have been identified in both simple and stratified epithelia, as well as cardiac myocytes and lymphoid tissue (Nuber, Schafer et al. 1995) (Schafer, Koch et al. 1994). In a simple (non-stratified) epithelium such as the colon, the only desmocollins expressed are DSC2 and DSG2 (Schafer, Koch et al. 1994; Nuber, Schafer et al. 1995). The expression of the other isoforms is mostly observed in stratified epithelia, such as the epidermis, oesophagus and cervix, whereby DSC3 and DSG3 predominate in the basal layers and DSC1 and DSG1 are expressed in the more superficial, terminally-differentiated layers (Theis, Koch et al. 1993), (Nuber, Schafer et al. 1996), (North, Chidgey et al. 1996; Chidgey, Yue et al. 1997) (Amagai, Koch et al. 1996) (Legan, Yue et al. 1994). At the amino acid level, the desmocollin and desmoglein isoforms within a species share 51-55% sequence identity and between different species, the individual isoforms appear to show up to 83% homology (Huber 2003).

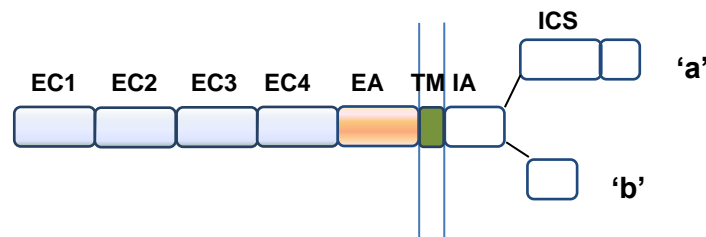
Desmosomal cadherins are transmembrane glycoproteins that consist of a transmembrane domain, which interacts with the desmosomal cadherins of the adjacent cell and a cytoplasmic portion, which interacts with the intermediate filament network through binding to plakoglobin, plakophilin and desmoplakin (Fig.1.3). The desmosomal cadherins share some structural homology with the classical cadherins, especially in the extracellular domain

(Wheelock and Johnson 2003). The transmembrane portion consists of five extracellular repeats, with the one that is most proximal to the membrane termed the extracellular anchor (Green and Gaudry 2000).

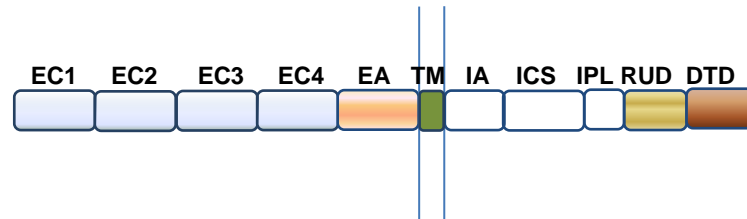
The desmocollins and desmogleins interact through the cell adhesion recognition sites in their extracellular domains (Runswick, O'Hare et al. 2001). The desmocollins and desmogleins bind in a heterotypic fashion (Chitaeu and Troyanovsky 1997; Marcozzi, Burdett et al. 1998) and at least one desmocollin and one desmoglein are required to confer desmosomal adhesion (Tselepis, Chidgey et al. 1998). Other studies have identified homophilic as well as heterophilic interactions, which appear to be isoform-specific (Syed, Trinnaman et al. 2002) (Nie, Merritt et al. 2011).

Fig 1.2 Structure of desmosomal components

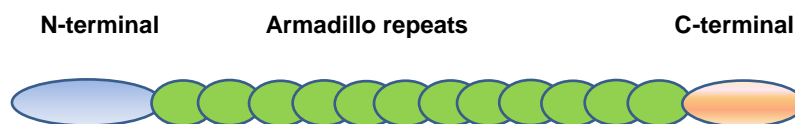
a. Desmocollin



b. Desmoglein



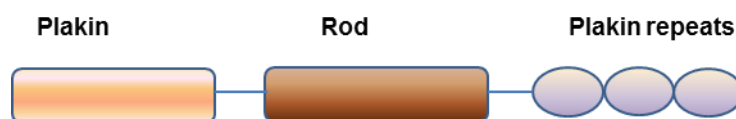
c. Plakoglobin



d. Plakophilin



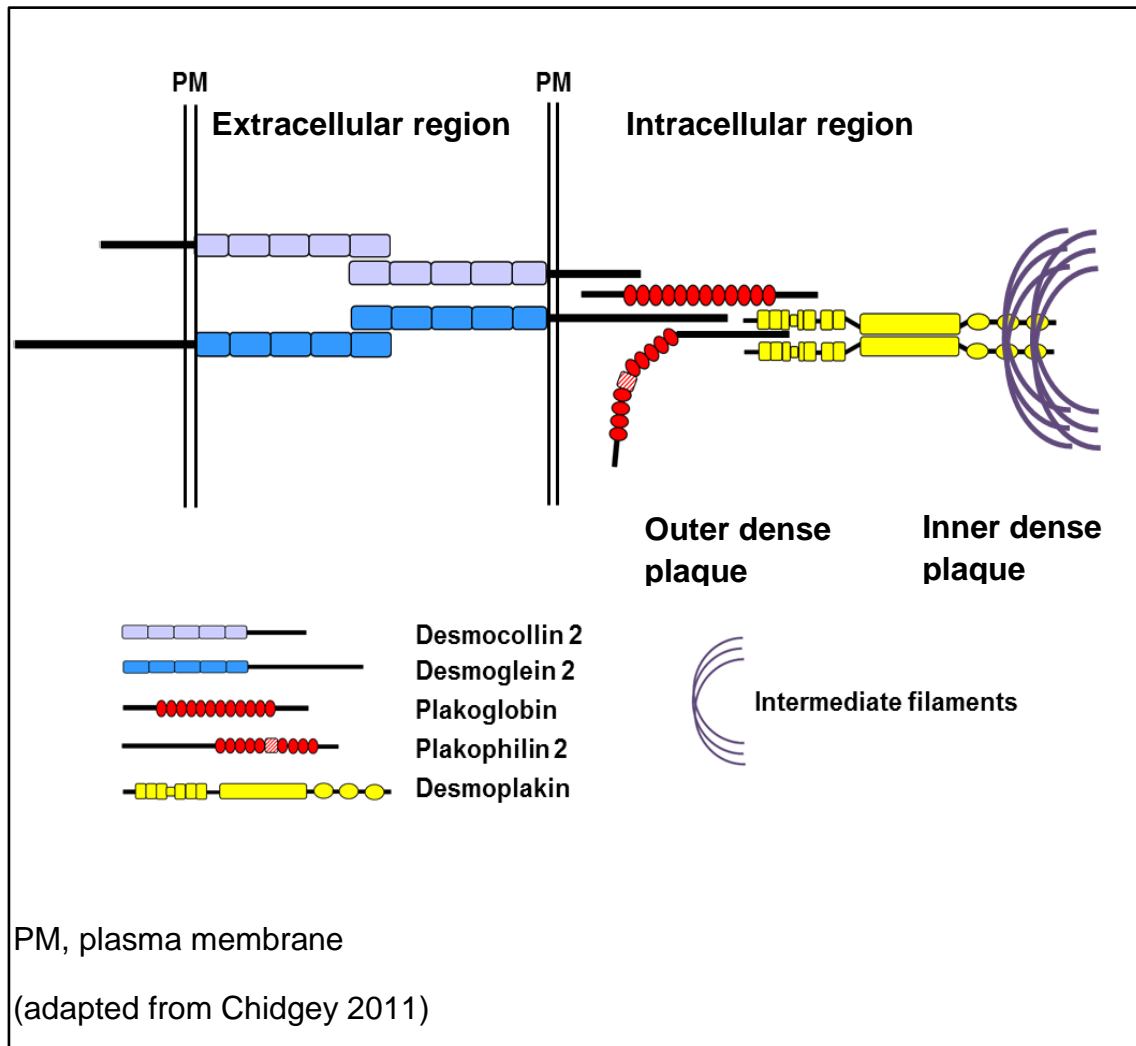
e. Desmoplakin



EC1-4, Extracellular repeat domain; EA, extracellular anchor; TM, transmembrane domain; IA, intracellular anchor; ICS, intracellular cadherin segment; IPL, intracellular proline-rich linker; RUD, repeat until domain, DTD, desmoglein specific domain. 'a' and 'b' indicate desmocollin splice variants. The blue box in the plakophilin sequence indicates a 61 amino acid sequence that introduces a bend in the Arm repeat domain.

Distinction arises between the desmocollins and desmogleins in the intracellular domain. They all contain an intracellular anchor domain adjacent to the membrane. The desmocollin cytoplasmic domain undergoes alternative splicing, to give two isoforms 'a' and 'b' (Fig 1.2). The 'a' isoform has an intracellular cadherin segment (ICS) which provides a binding site for plakoglobin (Trojanovsky, Eshkind et al. 1993; Mathur, Goodwin et al. 1994; Chitaev, Averbakh et al. 1998). The 'b' splice variant has a truncated ICS, so is unable to bind plakoglobin. The role of the DSC 'b' isoform is unknown at this time. As well as interacting with plakoglobin, the desmosomal cadherins bind plakophilins (Bonne, Gilbert et al. 2003; Gehmlich, Syrris et al. 2011). Desmogleins have a much more extensive cytoplasmic domain than the desmocollins (Fig 1.2), which has an additional intracellular proline-rich linker (IPL), a 29-amino acid repeat unit domain and a C-terminal glycine-rich terminal domain (Kowalczyk, Bornslaeger et al. 1999).

Fig 1.3 Structure of a desmosome



1.4.3 Armadillo proteins

The armadillo family members plakoglobin (also known as γ -catenin) and plakophilins 1-3 are cytoplasmic desmosomal proteins. They are characterised by their central domains, which consist of a series of imperfect 42-amino acid repeats, known as Arm-repeats. The Arm-repeat domain provides binding sites for both the desmosomal cadherins and desmoplakin (Trojanovsky, Chitaev et al. 1996; Kowalczyk, Bornslaeger et al. 1997; Huber 2003). Evidence for the essential role of both plakoglobin and the plakophilins in the structure of desmosomes comes from the plakoglobin- and plakophilin 2 null mouse models. Knockout of either plakoglobin or plakophilin 2 results in embryonic lethality at 12-16 days as a result of desmosomal disruption in cardiac cells, leading to cardiac rupture and death (Ruiz, Brinkmann et al. 1996; Achenbach, Brunner et al. 2003; Grossmann, Grund et al. 2004).

In addition to its structural function, plakoglobin also plays a role in cellular signalling. It shares significant homology with β -catenin, another armadillo family member, which has a well-established signalling role in the wnt pathway and consequently, colorectal cancer (Morin, Sparks et al. 1997; Morin 1999). Whereas β -catenin is usually restricted to adherens junctions, plakoglobin can incorporate into both adherens junctions and desmosomes. As a result, it is able to mediate communication between these two types of junction and promote epithelial adhesion (Getsios, Huen et al. 2004). Plakoglobin is involved in signalling when it is not tethered to the structure of either of these cell junctions. Like its homologue, β -catenin, plakoglobin is

able to activate TCF/LEF family of transcription factors, even in a β -catenin-deficient cell line (Maeda, Usami et al. 2003). In addition it has been shown to interfere with β -catenin proteasomal degradation (Salomon, Sacco et al. 1997), thus increasing the cytoplasmic β -catenin pool. However, plakoglobin does not have the same potency as β -catenin to activate the TCF/LEF pathway (Simcha, Shtutman et al. 1998) and may in fact have act as a negative regulator of β -catenin signalling (Miravet, Piedra et al. 2002). Plakophilins may also have a signalling role in the wnt pathway as over-expression of plakophilin 2 in colon carcinoma cells leads to an increase in β -catenin/TCF signalling (Chen, Bonne et al. 2002).

1.4.4 Desmoplakin

Desmoplakin (DSP) is one of the obligatory proteins required to mediate desmosomal adhesion. It has two isoforms (DPI and DPII) which arise through alternative splicing and differ only in the length of their rod domains. The N-terminal end of desmoplakin is able to bind to the armadillo proteins (Kowalczyk, Bornslaeger et al. 1997) whereas its C-terminal end interacts with the intermediate filament network (Kouklis, Hutton et al. 1994).

1.4.5 Regulation of desmosomal gene expression

Little is known about desmosomal gene expression but it is assumed to take place in a coordinated manner as all the desmosomal cadherin genes are tightly clustered together on chromosome 18 (Hunt, Sahota et al. 1999; Whittock, Hunt et al. 2000). CCAAT/enhancer binding proteins (C/EBPs)

have been shown to regulate desmocollin 1 and desmocollin 3 gene expression in keratinocytes (Smith, Zhu et al. 2004). In addition, CDX1 and CDX2 appear to control *DSC2* expression in colonic cells (Funakoshi, Ezaki et al. 2008) and p53 plays a role in the regulation of *DSC3* expression in breast cancer cells (Oshiro, Watts et al. 2003).

1.4.6 Desmosomes and disease

The importance of desmosomes in tissue integrity has been illustrated by knockout mouse models. *DSG2*, desmoplakin, and plakoglobin null mice (Bierkamp, McLaughlin et al. 1996; Ruiz, Brinkmann et al. 1996; Gallicano, Kouklis et al. 1998; Eshkind, Tian et al. 2002) all resulted in embryonic lethality, demonstrating severe heart and skin defects. The *DSC1* and *DSG3* knockouts were both compatible with life but exhibited severe skin changes (Koch, Mahoney et al. 1997; Chidgey, Brakebusch et al. 2001).

Both acquired and inherited disorders occur as a result of defective desmosomal adhesion. The skin blistering disorders pemphigus vulgaris and pemphigus foliaceus are autoimmune diseases with pathogenic antibodies directed against *DSG3* and *DSG1* respectively (Amagai, Koch et al. 1996; Koch, Mahoney et al. 1997; Stanley and Amagai 2006). The exfoliative toxin produced by *Staphylococcal aureus* in staphylococcal scalded skin syndrome cleaves *DSG1* in the suprabasal layers of the epidermis (Amagai, Matsuyoshi et al. 2000).

A number of conditions arise from inherited mutations in desmosomal genes. These generally affect either the heart or skin, and sometimes both (Chidgey 2011). For example mutations in any of the five desmosomal genes that are expressed in the heart (*DSC2*, *DSG2*, *JUP*, *PKP2* and *DSP*) can give rise to arrhythmogenic right ventricular dysplasia, a cause of sudden cardiac arrest and death, particularly in young adults (Awad, Calkins et al. 2008). Mutations in the genes encoding plakoglobin and desmoplakin can give rise to Naxos disease and Carvajal syndrome respectively, characterised by palmoplantar keratoderma, woolly hair and heart disease (Chidgey, 2011).

1.4.7 Desmosomes and cancer

Altered expression of various desmosomal components has been demonstrated in cancer. Loss of heterozygosity of chromosome 18q, in the region occupied by the desmosomal cadherin gene cluster, has been demonstrated in both head and neck cancers and oesophageal cancer (Karkera, Ayache et al. 2000; Takebayashi, Ogawa et al. 2000). Decreased expression of *DSG2* has been reported in gastric cancer (Biedermann, Vogelsang et al. 2005). Similarly, loss of expression of *DSC2* in colon cancer (Khan, Hardy et al. 2006) and *DSC3* in breast cancer (Oshiro, Kim et al. 2005) has been reported. Loss of individual desmosomal cadherins has been shown to correlate with invasion, metastasis and survival in oral cancers (Shinohara, Hiraki et al. 1998), head and neck cancers (Wong, Cheang et al. 2008) and pancreatic cancer. Conversely, increased *DSG2* expression has been observed in skin cancers investigated by microarray (Brennan and Mahoney 2009).

Other desmosomal components exhibit altered expression in cancer. Plakophilin 1 expression is lost in oesophageal squamous cell carcinoma (Sobolik-Delmaire, Katafiasz et al., 2007) and plakophilin 3 loss has been demonstrated in colorectal cancer (Aigner, Descovich et al. 2007). Reduced expression of desmoplakin has been correlated with the progression of a variety of cancers including breast cancer (Davies, Gee et al. 1999).

Plakoglobin appears to have both oncogenic and tumour suppressor effects. As described in section 1.4.3, plakoglobin may have a role in the wnt signalling pathway. Overexpression of plakoglobin promotes cell proliferation, through the activation of c-myc (Kolligs, Kolligs et al. 2000) but also acts to inhibit apoptosis by induction of the pro-survival gene *bcl-2* (Hakimelahi, Parker et al. 2000). However, LOH at the plakoglobin gene locus on chromosome 17q has been identified in breast and ovarian tumours (Aberle, Bierkamp et al. 1995) and restoration of plakoglobin in bladder cancer cell lines was able to reduce proliferation and migration (Rieger-Christ, Ng et al. 2005). This and other evidence indicates that plakoglobin plays an important role in cancer, although this may be related more to its signalling capacity than its structural role. It has been suggested that sequestration of plakoglobin in desmosomes may serve to regulate the non-junctional pool of plakoglobin available for signalling (Chidgey and Dawson 2007).

The epithelial-mesenchymal transition (EMT) in cancer allows epithelial cells to gain mesenchymal characteristics i.e increased motility and invasiveness. In order for this to take place, intercellular adhesion is downregulated. Dissociation of both adherens junctions and desmosomes is central feature of EMT. The EMT regulators Slug, Snail and zinc finger binding proteins ZEB1 and ZEB2 have been shown to cause desmosomes to dissociate (Savagner, Yamada et al. 1997; Vandewalle, Comijn et al. 2005). In keeping with the role of EMT in normal cell differentiation and repair, wounding of the epithelium results in Slug-mediated desmosome-dissociation, which allows desmosomes to migrate from the wound edges to fill the defect (Savagner, Kusewitt et al. 2005).

Desmosomal proteins have also been shown to be targeted in apoptosis (Weiske, Schoeneberg et al. 2001). Also, *DSC3* has been shown to be a p53 target gene in breast cancer (Oshiro, Watts et al. 2003). More recently the desmosomal related protein PERP has also been implicated in cancer. It is activated by p53 and p63 to mediate their tumour suppressor function in skin squamous cell carcinoma (Dusek and Attardi 2011), although the role of PERP in desmosomes has not been fully elucidated.

A potential role for desmosomes in cell proliferation and cancer has emerged from transgenic mouse studies. This misexpression of the desmogleins DSG2 and DSG3 in inappropriate layers of the epidermis results in a hyper-

proliferative phenotype that resists apoptosis (Merritt, Berika et al. 2002; Brennan, Hu et al. 2007). Similarly misexpression of desmocollin 3 in the epidermis results in altered differentiation (Hardman, Liu et al. 2005). DSG2 misexpression activates multiple signalling pathways, including PI-3K/AKT, mitogen-activated protein kinase (MAPK) and NF-KB pathways (Hardman, Liu et al. 2005; Brennan, Hu et al. 2007) whereas misexpression of DSC3 increases β -catenin transcriptional activity (Hardman, Liu et al. 2005). Furthermore, deletion of the desmoplakin gene in a pancreatic cancer mouse model results in increased invasive potential (Chun and Hanahan 2010).

A role for desmosomes in cancer has been inferred from experiments in cultured cells. For example, transfection of desmosomal proteins into non-adhesive fibroblasts was able to inhibit the invasive behaviour of the cells (Tselepis, Chidgey et al. 1998). By contrast, knockdown of *DSC2* in colonic adenocarcinoma-derived cells promotes cell proliferation and transformation through activation of the AKT/ β -catenin signalling (Kolegraff, Nava et al. 2011).

1.5 Aims

To determine the role of desmosomal cadherins in colorectal cancer, three lines of investigation were pursued:

1. The role of the desmocollins in colorectal cancer was investigated by measuring their expression levels in a series of matched normal and tumour samples by western blotting and real-time polymerase chain reaction (PCR). As part of this study, desmoglein mRNA levels in the matched normal and tumour samples were also measured by real time rt-PCR.
2. A cell culture model was established to determine the role of desmosomal cadherins in cell behaviour. In these experiments antisense cDNAs were used to knockdown desmocollin 2 and desmoglein 2 mRNA levels in cultured LS174T colon cancer cells.
3. Little is known of how desmosomal cadherin gene expression is regulated in the colon. Thus, reporter gene assays were carried out in cultured Caco2 colon cancer cells to identify transcription factors that are important in the regulation of desmocollin 2 and desmoglein 2 gene expression in colonic epithelial cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Human tissue samples

Specimens were collected from patients undergoing surgery for colorectal cancer at the Queen Elizabeth Hospital, Birmingham with appropriate consent and in accordance with local ethics committee guidelines. Matched samples of colorectal cancer and normal colonic tissue (from macroscopically normal tissue within the surgical specimen) were harvested and immediately frozen and stored in liquid nitrogen at -80°C (Table 2.1). Samples were used from 21 patients, of which 13 were male and 8 female. The median age was 76 years (interquartile range 62-80 years).

In addition, tissue was obtained from patients undergoing surgery for non-neoplastic conditions to provide control samples. Normal Control patient 1 (NC1) underwent a total colectomy for distal ulcerative colitis limited to the recto-sigmoid and refractory to medical treatment. Specimens were taken from the caecum, as far away from the involved tissue as possible. Normal Control patient 2 (NC2) underwent an anterior resection of the rectum for diverticular disease with covering ileostomy formation. The excised disc of skin from the ileostomy site provided a normal skin control. Both samples were taken with the appropriate consent and were stored in liquid nitrogen at -80°C .

Table 2.1 Clinicopathological features of tumour samples

		Number of patients
Dukes Stage	A	1
	B	13
	C	5
	D	2
T Stage	1	1
	2	0
	3	12
	4	8
Location	Right	10
	Transverse	2
	Sigmoid	4
	Rectum	5

2.2 Cell lines

The following cell lines were obtained from the American Type Culture Collection (ATCC):

LS174T is a colon adenocarcinoma cell line derived from 58 year old Caucasian female with a Dukes B adenocarcinoma (Tom, Rutzky et al. 1976).

Caco2 is a colonic adenocarcinoma cell line derived from a 72 year old Caucasian male patient (Fogh, Fogh et al. 1977).

HT29 is a colonic adenocarcinoma cell line, originally isolated from a 44 year old female patient (Fogh, Fogh et al. 1977).

MCF7 is a human breast adenocarcinoma cell line, derived from the pleural effusion of a 69 year old Caucasian female patient (Soule, Vazquez et al. 1973).

Primary Normal Human Epithelial Keratinocytes (NHEK-A cells) were obtained from TCS Cellworks.

2.3 Tissue culture

LS174T cells were cultured in Dulbecco's Modified Essential Medium (DMEM) (Sigma) supplemented with 10% foetal bovine serum (Sigma), 1% non-essential amino acids (Invitrogen), 2mM L-Glutamine (Sigma) and 5µl/ml bovine insulin (Sigma).

Caco2 and MCF7 cells were cultured in Eagle's Minimum Essential Modified Medium (EMEM, Sigma), supplemented with 20% foetal bovine serum, 1% non-essential amino acids, 1% L-Glutamine and 5µl/ml bovine insulin.

HT29 cells were cultured and maintained in RPMI medium (Sigma), supplemented with 10% foetal bovine serum and 1% L-Glutamine.

Primary Normal Human Epithelial Keratinocytes (NHEK-A cells) were cultured in Keratinocyte Growth Medium (KGM) (TCS Cellworks). All cells were maintained at 37°C in 5% CO₂.

2.4 RNA and protein extraction

Tumour and normal colonic mucosa specimens were cut into 5mm x 10mm pieces and homogenised in 0.75ml of TRI Reagent (Helena Biosciences). RNA and protein was prepared according to the manufacturer's protocol. RNA concentration was determined by the measurement of absorbance at 260nm in a spectrophotometer. Protein concentration was measured using the Bio-Rad Protein Assay (Bio-Rad) according to the manufacturer's instructions with Bovine Serum Albumin (BSA) as standard.

To prepare RNA from human skin 10mm² pieces of skin were first chopped with a scalpel whilst frozen, into smaller (1mm²) pieces. These were added to 2ml of guanidinium thiocyanate (GTC) buffer (Sambrook, Fritsch et al. 1989). 200µl 2M sodium acetate (pH 4.0), 2ml water saturated phenol and 400µl chloroform was added and the mixture vortexed for 10sec, incubated on ice for 10min, and centrifuged for 20min at 10,000rpm and 4°C. The upper aqueous layer was then added to 2ml isopropanol and stored at -20°C for >1 hour. The sample was centrifuged (20min at 10,000rpm and 4°C) and the pellet dissolved in 400µl nuclease free water. 20µl 3M sodium acetate (pH 6.0) and 1ml ice-cold ethanol were added and the sample stored at -70°C for >1h. The sample was then centrifuged for 20min at 13,000rpm and 4°C. The pellet was washed with 70% ethanol, dried and dissolved in 50µl nuclease free water. RNA concentration was determined by the measurement of absorbance at 260nm in a spectrophotometer. RNA quality was checked by denaturing formaldehyde polyacrylamide gel electrophoresis (section 2.5.3).

RNA was extracted from cultured cells using RNA-Bee (Biogenesis) Cells were grown to 70% confluence and washed in phosphate buffered saline. For each 100mm plate, 2ml of reagent was used to lyse the cells. Chloroform (0.25ml) was added and the mixture vortexed and incubated on ice for 5 min. Samples were then centrifuged for 15min at 1,000 rpm and 4°C. RNA was precipitated by mixing the upper aqueous phase with an equal volume of isopropanol, followed by incubation at room temperature for 10 min and centrifugation at 10,000rpm for 10 min at 4°C. The RNA pellet was washed in 70% ethanol and allowed to dry. RNA was dissolved in 50µl nuclease-free water.

2.5 Analysis of RNA

2.5.1 Quantitative reverse-transcriptase mediated real time PCR

Reverse transcription was performed using the First Strand cDNA Synthesis kit (Roche) with 1µg of RNA per reaction to yield 20µl cDNA. Real-time PCR primers (Table 2.2) were designed using Primer Express software, version 1.5 (PE Applied Biosystems, Cambridge, UK) and obtained from Alta Biosciences (University of Birmingham, Birmingham, UK). Real-time PCR was performed using the SYBR Green method in the ABI Prism® 7700 Sequence Detector. The Sensimix (dT) kit (Quantace) was used together with primers at 300nM concentration and 1µl of cDNA in each 25µl reaction. An epithelial cell specific gene, cytokeratin 8 (*CK-8*) was used as an internal control. Thermal cycling conditions were as follows: 50°C for 2min, 95°C for 10min, then 40 cycles of 95°C for 15sec and 60°C for 1min. All samples were run in triplicate, together with positive and negative controls. Interpretation was performed using the comparative threshold (C_T) method with ABI Sequence Detection System software and Microsoft Excel.

Table 2.2 Primers used for real-time PCR

Gene	Accession number	Sequence of Forward (F) and Reverse (R) primers	Size of PCR product (bp)
<i>DSC1</i>	NM_024421	F: TGCCAAACACTCCTCACTCAAA R: TTCAATGGCTTGACAACACACA	124
<i>DSC2</i>	NM_024422	F: CGGAGATTGTTGCGGTTGA R: GGAAAGACGTGCTGCTGTATCA	134
<i>DSC3</i>	NM_001941	F: GGCTGTTGCGCTGTCTGATAA R: GCCCAAGGAATTCTCTTGCA	202
<i>DSG1</i>	NM_001942	F: CGGAAACGGAGCCAAAGATT R: TCAAAGCCAGCTGCACTACGA	156
<i>DSG2</i>	NM_001943	F: AAACAGTGGCCCTTTCAGTTTC R: GTTGCAGCAGCACACTGGTACT	101
<i>DSG3</i>	NM_001944	F: GTCTCCTGCTGCTGCTGTTG R: TCCTTCTGAGCCATCAGGAAGT	113
<i>DSG4</i>	AY227350	F: GCAAGGAAGAGGAGCTCTACCA R: GCATACGCTTTCTCCGAGAAGT	101
<i>CK-8</i>	NM_002273	F: GATCGCCACCTACAGGAAGCT R: ACTCATGTTCTGCATCCCAGACT	70

2.5.2 Semi-quantitative reverse transcriptase-mediated PCR

RNA was extracted from cultured cell lines and cDNA synthesised as described (section 2.5.1). Semi-quantitative PCR was performed using the Expand High Fidelity PCR System (Roche) and the following thermal cycling conditions: 94°C for 5min, followed by 25 cycles of 94°C for 30seconds, 57°C for 30 seconds and 72°C for 2min. The primers shown in Table 2.3 were used to amplify desmocollin and desmoglein DNA.

Table 2.3 Primers used for semi-quantitative RT-PCR

Gene	Sequence of Forward (F) and Reverse (R) primers	Size of PCR Product (bp)
<i>DSC1</i>	F: TTGGATACACACTGGACC R: CCAGAAAGAATTGAAAAGGTGG	400
<i>DSC2</i>	F: AGAAGCCTGGATAGAGAGG R: CCAAGGATTGGCGGTGGA	460
<i>DSC3</i>	F: GCACTCCTGCAGCCCAAT R: TACTCATCCAACTCAGTGTC	600
<i>DSG1</i>	F: GACCTGCTAGCTGTTGATTC R: GCTGGTATCATAGTTAGTGG	240
<i>DSG2</i>	F: CCATCACTGGCACAGTCCT R: AGGAGTCATGCTGTGCTTC	360
<i>DSG3</i>	F: CAATCACAGCTGAGGTTCTG R: CAGTGTCAAGCTGCGTGG	350
<i>DSG4</i>	F: GGCATCTACACAGAGGACAT R: GTTGATGTCTGCGTCAGCGT	522

2.5.3 Formaldehyde agarose gel electrophoresis

Formaldehyde agarose gel electrophoresis was performed according to the method of Sambrook, Fritsch et al (1989). RNA was precipitated using lithium chloride, resuspended in RNA sample buffer and incubated at 55°C in a heat block for 30min. The samples were then cooled on ice for 10min prior to adding 3µl of formaldehyde gel-loading buffer (Sambrook, Fritsch et al. 1989) with ethidium bromide (1mg/ml). Samples were loaded on formaldehyde denaturing agarose gels alongside a High Range RNA ladder (Fermentas) and run at 50V for 2 hours (to check RNA quality) or 4 hours (for northern blotting). 2µg of RNA was used when checking RNA quality, whereas 10-20µg of RNA per lane was used for northern blotting. RNA was visualised using a transilluminator and UV light.

2.5.4 Northern blotting

RNA was resolved by denaturing formaldehyde gel electrophoresis as described (section 2.5.3). The gel was washed twice in 10x SSC buffer (Sambrook, Fritsch et al. 1989) for 30min with agitation. Samples were transferred onto Amersham Hybond™-N (GE Healthcare) using 10x SSC buffer. The membrane was then washed in 2x SSC buffer, dried and baked at 80°C for 2 hours to facilitate crosslinking of the RNA to the membrane. This was then placed in a hybridisation bottle with 10ml of hybridisation buffer and incubated at 42°C for 30min. The hybridisation probe was denatured by heating to 95°C for 5min then added to the hybridisation bottle and incubated

at 42°C for at least 4 hours. The filters were then removed, washed and exposed to X-ray film at -80°C. The X-ray film was developed after 4 days.

2.5.5 Northern blot hybridisation probes

2µg of DNA (made up to 10µl with water) was heated to 95°C for 3min and then cooled on ice for 10min. Labelling of the probe DNA was achieved using the Radiolabelling Primer Kit (Roche), with 2µl Solution 6, 1µl of each of dATP, dTTP and dGTP, 4µl [α -³²P]-dCTP (3000Ci/mmol) (ICN Biomedical Research Products) and 1µl of Klenow enzyme. The mixture was incubated at 37°C for 45min. Unincorporated nucleotides were removed using the QIAquick Nucleotide Removal Kit (Qiagen). The labelled probe was stored at -80°C in a lead-lined box until needed.

2.6 Western blot analysis

Protein samples (10µg) was denatured by adding 6M urea and 5µl of 2x sample buffer (Sambrook, Fritsch et al., 1989) (to a total volume of 10µl) and incubating at 95°C for 5min. Proteins were resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using standard techniques (Sambrook, Fritsch et al., 1989). Proteins were transferred onto polyvinylidene difluoride (PVDF) membrane (Hybond P, Amersham Biosciences). Blots were blocked in 5% non-fat dried milk in Tris-buffered saline (TBS) containing 0.1% Tween (TTBS). Blots were probed with primary antibodies (Table 2.4), followed by either horseradish peroxidase (HRP) conjugated anti-rabbit IgG (1:10,000, Sigma) or HRP-conjugated anti-mouse IgG (1:1000, Jackson ImmunoResearch) secondary antibodies (as appropriate). Proteins were visualised with an enhanced chemiluminescence (ECL) substrate (Amersham; as specified by the manufacturer) and autoluminography.

In order to obtain a quantitative analysis, the western blots were assessed using Image J software (v.1.33, National Institute of Health, USA <http://rsbweb.nih.gov/ij>) which allowed comparison of the protein content between normal and tumour samples after normalisation with cytokeratin 8.

Table 2.4 Primary antibodies used for western blotting

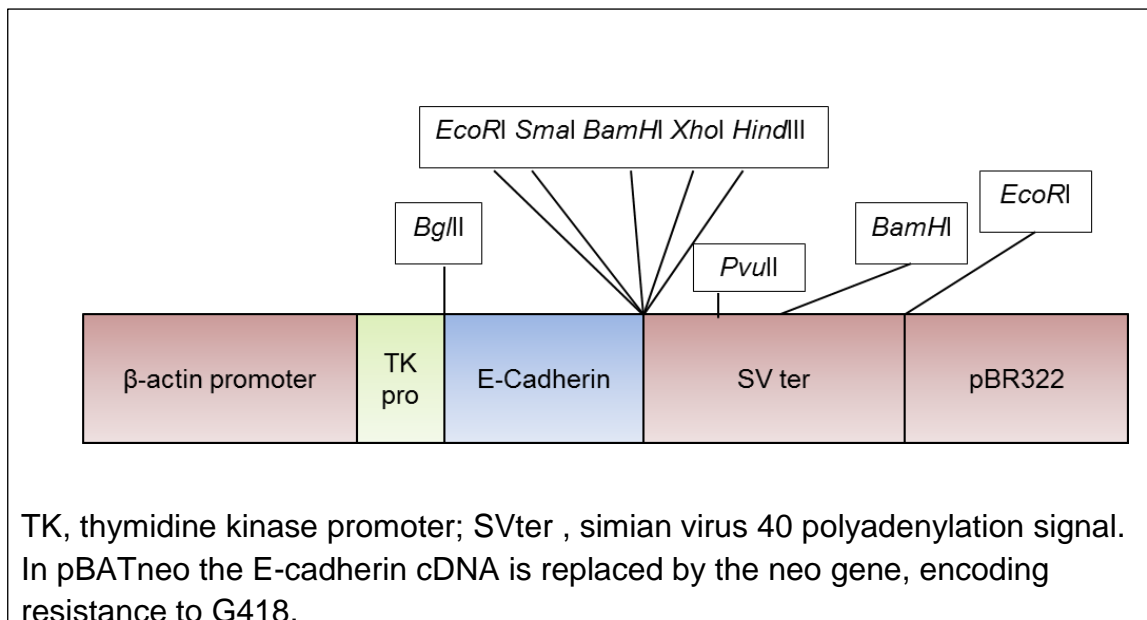
Protein	Antibody	Dilution
DSC1	JCMC, (North, Chidgey et al. 1996)	1:10,000
DSC2	610120, Progen	1:1000
DSC3	U114, Progen	1:50
DSG2	33-3D, (Vilela, Hashimoto et al. 1995)	1:50
Cytokeratin 8	C51, Zymed	1:200

2.7 Plasmids

2.7.1 pBATEM2 vector

The eukaryotic expression vector pBATEM2 (Fig 2.1) was provided by M Takeichi, Kyoto University, Japan. It is based on the plasmid pBR322 and contains DNA encoding mouse E-cadherin downstream of a composite promoter consisting of chicken β -actin and herpes simplex virus thymidine kinase DNA, and upstream of simian virus 40 polyadenylation signals (Nose, Nagafuchi et al. 1988). Human *DSC2* and *DSG2* antisense cDNA was cloned into the *Bgl*II and *Hind*III sites of pBATEM2 and the resulting plasmids used to stably transfect LS174T cells (see Chapter 4). As pBATEM2 does not contain a selectable marker, LS174T cells were co-transfected with pBATneo (also provided by Dr Takeichi), which confers resistance to the antibiotic G418.

Fig 2.1 pBATEM2.

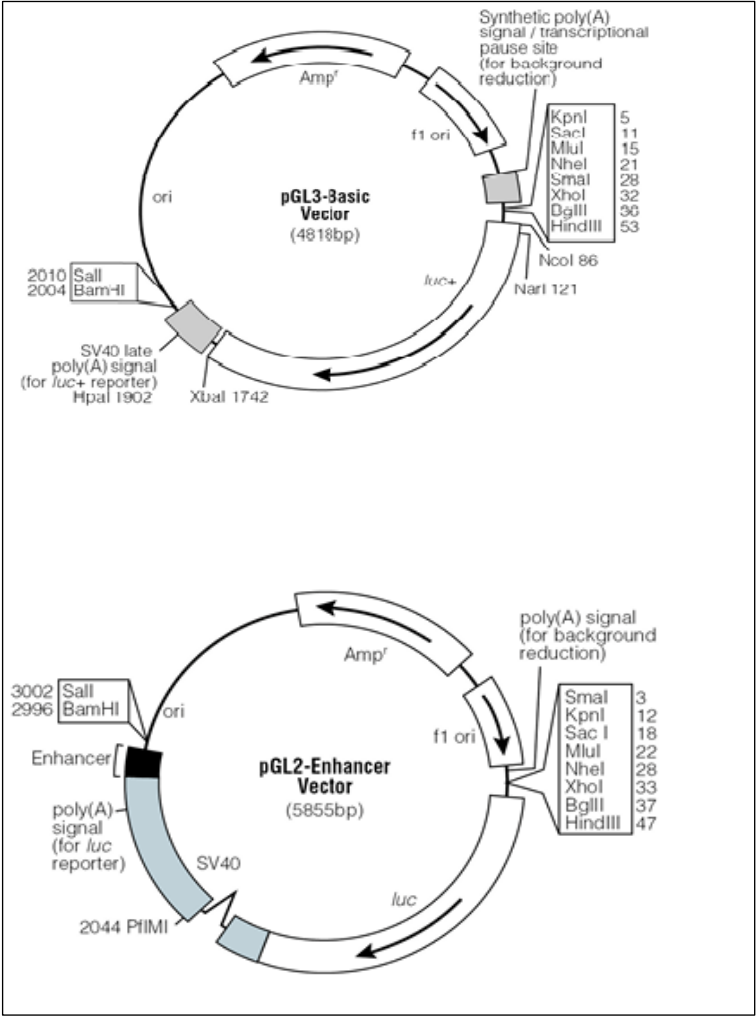


2.7.2 pGL2 and pGL3 reporter vectors

Luciferase reporter vectors pGL3-Basic and pGL3-Enhancer (Fig 2.2) were obtained from Promega. pGL3-Basic contains a firefly luciferase reporter gene (*luc*) downstream of a multiple cloning site (MCS). It lacks enhancer elements and is suitable for the analysis of strong promoters. pGL2-Enhancer also contains *luc* downstream of a MCS. It contains an SV40 enhancer and so is suitable for analysing weaker promoter elements. Fragments of the human *DSC2* and *DSG2* promoters were inserted into the *KpnI* and *XhoI* sites of pGL2-Basic and pGL2-Enhancer reporter plasmids and used in transient transfection of Caco2 cells (see Chapter 5).

Plasmids pGL2E-*Dsc1*(0.4) and pGL3B-*Dsc3*(0.3) were provided by Dr MA Chidgey (University of Birmingham). Plasmid pGL2E-*Dsc1*(0.4) contains bases -1 to -417 of the mouse *Dsc1* promoter in pGL2-Enhancer and plasmid pGL3B-*Dsc3*(0.3) contains bases -1 to -293 of the mouse *Dsc3* promoter in pGL3-Basic (Smith, Zhu et al. 2004).

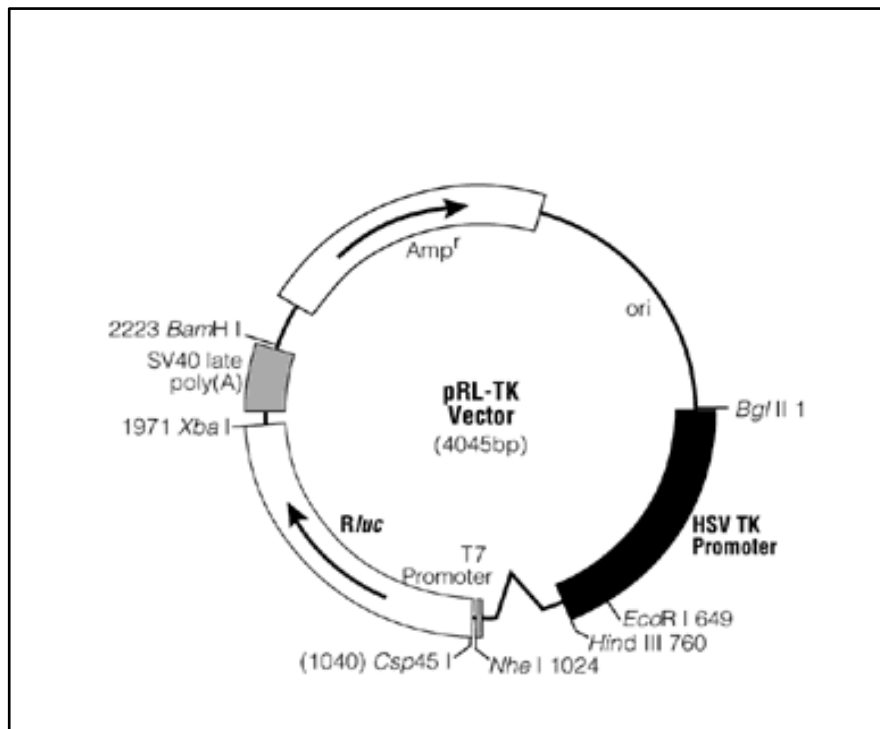
Fig 2.2 pGL3-Basic and pGL2-Enhancer plasmids.



2.7.3 pRL-TK

Vector pRL-TK (Promega) (Fig 2.3) was used to monitor transfection efficiency in luciferase reporter assays. It contains the herpes simplex virus thymidine kinase (HSV-TK) promoter upstream of a gene encoding *Renilla* luciferase.

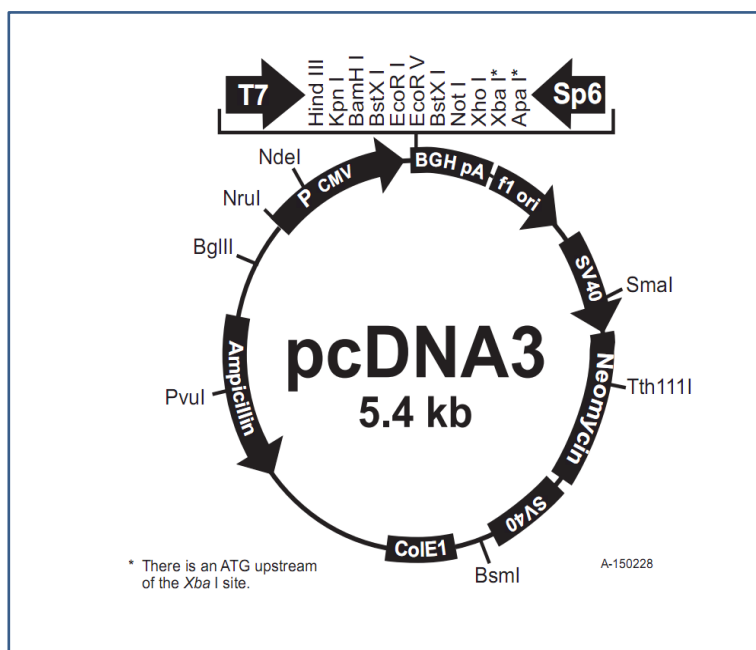
Fig 2.3 pRL-TK.



2.7.4 pcDNA3 based plasmids

The eukaryotic expression vector pcDNA3 (Fig 2.4) was obtained from Invitrogen. It contains a multiple cloning site downstream of the cytomegalovirus (CMV) promoter and upstream of Bovine Growth Hormone (BGH) polyadenylation signal. pcDNA3 based plasmids encoding a variety of transcription factors were co-transfected into Caco2 cells with pGL3-Basic reporter constructs (see Chapter 5). pcDNA3 plasmids encoding full-length rat C/EBP α and C/EBP β were provided by Dr RC Smart of North Carolina State University. pcDNA3 plasmids encoding mouse C/EBP δ , C/EBP ζ , c-fos, c-jun, JunB, AP2 α , AP2 γ , AP4 and p53 were provided by Dr MA Chidgey. Plasmids encoding full-length human plakoglobin and Lef1 were also provided by Dr Chidgey. A pcDNA3 plasmid encoding a stable S37A mutant version of full-length human β -catenin, which is resistant to degradation, was provided by Dr SW Byers of Georgetown University.

Fig2.4 pcDNA3



2.8 DNA manipulation

2.8.1 Restriction enzyme digests

Restriction enzyme digests were performed using enzymes from a variety of sources according to the manufacturer's instructions. Usually digests contained 1µl restriction enzyme (10 units), 1µl of the appropriate buffer (10x), 1µl BSA, 5µl of DNA and water (in a total volume of 10µl). Digests were incubated for 1h at 37°C.

2.8.2 Agarose gel electrophoresis

Agarose gels were prepared and run according to the method of Sambrook, Fritsch et al. (1989), using 1% agarose (Sigma) dissolved in 1 x TAE buffer (40mM-Tris acetate, 1mM-EDTA, pH7.8). DNA samples were resolved alongside λ HindIII markers (Roche). Gels were stained by soaking in ethidium bromide solution (5µg/ml) for 10-20min and DNA was visualised on a UV transilluminator.

2.8.3 DNA isolation by electroelution

DNA samples were resolved by agarose gel electrophoresis. The agarose containing the required DNA was excised from the gel under UV light and placed in dialysis tubing (Medicell International Ltd) with 1x TAE buffer. The ends of the tubing were clamped, the tubing placed in an electrophoresis tank and a current of 90V applied for 45min. The current was reversed for 30sec

and the liquid aspirated from the dialysis tubing. The sample was extracted with phenol and chloroform and the DNA recovered by ethanol precipitation.

2.8.4 Ethanol precipitation of DNA

DNA was precipitated using one half-volume of 8M-ammonium acetate and 2 volumes ethanol. After vortexing, the sample was placed at -80°C for >30 min, and then centrifuged at 13,000rpm for 10min. The DNA pellet was washed with 70% ethanol, dried and redissolved in the required volume of TE buffer (10mM Tris.HCl, 1mM-EDTA, pH8.0).

2.8.5 Ligation reactions

DNA fragments were ligated using T4 DNA ligase (200 units) and ligase buffer (both from New England Biolabs) in a 10µl reaction. Ligation reactions were carried out at room temperature for 16h.

2.8.6 Transformation of competent bacteria with DNA

Competent *E.Coli* XL1-Blue cells were prepared using standard techniques (Sambrook, Fritsch et al., 1989). DNA was transformed into these cells using the heat shock method of Sambrook, Fritsch et al (1989). Briefly, the host cells were thawed in a 37°C water bath and allowed to rest on ice for 5min. Plasmid DNA was added, the cells left on ice for a further 30 minutes and then heat shocked (42°C for 2min). 1ml of LB medium (Sambrook, Fritsch et al., 1989) (without ampicillin) was added and the cells incubated at 37°C for 1h. Cells were pelleted by low speed centrifugation, resuspended in 100µl of

LB medium, spread on LB/agar plates (1% agar in LM medium with 50µg/ml ampicillin) and incubated overnight at 37°C.

2.8.7 Plasmid DNA isolation

For small scale Mini-Prep DNA isolation, single colonies were picked from plates and incubated in 5ml LB medium (with 50µg/ml ampicillin) at 37°C, overnight in a shaking incubator. Plasmid DNA was extracted from 1.5ml of each culture using the alkaline lysis method (Sambrook 1989). For larger scale Midi-Prep DNA isolation 1ml of an overnight culture (as above) was added to 100ml of fresh LB medium (with 50µg/ml ampicillin) and incubated at 37°C overnight (with agitation). The Qiagen Plasmid Midi Kit was used to prepare the DNA from these cultures according to manufacturer's protocol. The recovered DNA was dissolved in 100µl TE buffer. DNA concentration was measured by spectrophotometry at 260nm.

2.8.8 DNA sequencing

The BigDye Terminator® v3 cycle sequencing kit (Applied Biosystems) was used with 500ng of DNA and 3pmol of primer per reaction. Thermal cycling conditions were as follows: 96°C for 3min, then 25 cycles of 96°C for 10sec, 50°C for 5 sec, and 60°C for 4min. Reaction products were precipitated using 2.5µl 0.2M EDTA and 64µl ethanol and recovered by centrifugation at 13000rpm for 10min. The pellet was washed with 70% ethanol (250µl), dried and dissolved in Hi-Di Formamide (Applied Biosystems). Samples were

analysed on ABI PRISM® DNA Analyser (Applied Biosystems) in the Functional Genomics Laboratory, University of Birmingham. Data were analysed using Chromas 1.45 sequence analysis software and the BLAST tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

2.9 Cloning of *DSC2* and *DSG2* antisense DNA

To produce *DSC2* antisense constructs for knockdown experiments (Chapter 4) fragments of *DSC2* cDNA, 824 and 785bp in length, were obtained by restriction enzyme digestion of a full length human *DSC2* cDNA clone (provided by T Magee, Imperial College, London). These were cloned into the pBAT backbone, derived from pBATEM2 (section 2.7.1). The procedures used are described in detail in Chapter 4. To produce *DSG2* antisense constructs fragments of *DSG2* cDNA were amplified by RT-PCR (section 2.5.2) using RNA isolated from human skin as starting material. The primers used are given in Table 2.4 were used. Fragments of *DSG2* cDNA, 825 and 811bp in length, were amplified using this procedure and cloned into the T-vector pDK101 (Kovalic, Kwak et al. 1991), sequenced to ensure that no unexpected mutations had been introduced during PCR amplification and released by restriction enzyme digestion. Again, the fragments were cloned in the antisense orientation into the pBAT backbone using the procedures described in chapter 4.

Table 2.5. Primers used to amplify the *DSG2* antisense fragments

PCR fragment	Sequence of Forward (F) and Reverse (R) primers
DSG2AS1	<p>F: AAG<u>TCGAC</u>CTTGCAGAAGAAAGAGGACTC <i>Sal</i>I</p> <p>R: TTGGATCCACTGAAGTCAAGATTCTTCAT <i>Bam</i>H1</p>
DSG2AS2	<p>F: AAG<u>TCGAC</u>GAAGCACAGCATGACTCCTA <i>Sal</i>I</p> <p>R: TTGGATCCAGAGTGAATGTGAAGCTGT <i>Bam</i>H1</p>

Restriction enzyme sites used for cloning are underlined.

2.10 Cloning of the human *DSC2* and *DSG2* promoters

Yeast artificial chromosome (YAC) clones 14E-B5 and 9G-C3, containing *DSC2* and *DSG2* promoter DNA respectively, were obtained from MRC geneservice. Clones were streaked out on LB/agar plates, containing ampicillin (50µg/ml) and tetracycline (12.5µg/ml), and single colonies picked and expanded. YAC DNA was isolated using the supplier's instructions.

Two fragments of the human *DSC2* promoter, bases -1 to -999 and -1 to -563, were amplified by PCR using the oligonucleotides in table 2.6. Similar sized

fragments of the human *DSG2* promoter were amplified using the same methodology. 50ng of YAC DNA was used per reaction. The Expand High Fidelity PCR System (Roche) was used with the following thermal cycling conditions: 94°C for 5min, followed by 25 cycles of 94°C for 30sec, 57°C for 30sec and 72°C for 2min.

Table 2.6 Primers used to isolate *DSC2* and *DSG2* promoter fragments.

Promoter fragment	Sequence of Forward (F) and Reverse (R) primers
<i>DSC2</i> -1 to -999	F: AAGGTACCGAACTCTCCACTGGATGTTCA <i>KpnI</i> R: AAGAGCTCCCTCTCCCGAGCCCCGTCCAG <i>XhoI</i>
<i>DSC2</i> -1 to -563	F: AAGGTACCGGCTCAGACCTCGCTCTGTAA <i>KpnI</i> R: AAGAGCTCCCTCTCCCGAGCCCCGTCTGAC <i>XhoI</i>
<i>DSG2</i> -1 to -1061	F': AAGGTACCTGAACCCTTAAGTTCTAGACACTGC <i>KpnI</i> R': AAGAGCTCGCGTGGGAGCGGAGGCGGAGG <i>XhoI</i>
<i>DSG2</i> -1 to -501	F': AAGGTACCTTGGTCCCGCAGGACTCTGCT <i>KpnI</i> R': AAGAGCTCGCGTGGGAGCGGAGGCGGA <i>XhoI</i>

2.11 Transfection of cultured mammalian cells

2.11.1 Stable transfection of antisense plasmids into LS174T cells

To obtain stable LS174T transfectants expressing antisense cDNA (Chapter 4), pBAT plasmids (section 2.7.1) containing either *DSC2* or *DSG2* antisense cDNA (100µg) were co-transfected into the cells with the vector pBATneo (10µg). Control cells were transfected with pBATneo alone. LS174T cells were plated onto 9cm tissue culture dishes and grown until 70% confluent. The cells were rinsed with phosphate buffered saline and DNA added in the form of a calcium phosphate precipitate using standard methodologies (Sambrook, Fritsch et al. 1989). The precipitate was allowed to settle on the cells for 20min and growth medium (section 2.3) added. After 48h the growth medium was supplemented with 800µg/ml G418 (Sigma). Surviving cell clones were picked after 2-3 weeks, expanded and screened by northern blotting, real-time PCR and western blotting (Chapter 4).

2.11.2 Transient transfection of luciferase reporter plasmids into Caco2 cells

Caco2 cells were grown to 70% confluence in 24 well plates in normal growth medium (section 2.3). Transient transfections were performed in serum free EMEM medium using Lipofectamine reagent (Invitrogen) as described by the manufacturer. In standard *DSC2* and *DSG2* reporter assays, 500ng of reporter plasmid was added per well, and transfection efficiency was monitored using plasmid pRL-TK (50ng). Cells were incubated in the transfection mixture for 4h in 5% CO₂ at 37°C. The transfection mixture was then aspirated from the cells and replaced with normal growth medium and

cells incubated for a further 48h. After 48h growth medium was removed and cells lysed using Passive Lysis Buffer (Promega). Lysates were stored at -70°C. Each experiment was performed in triplicate on at least two occasions to demonstrate reproducibility.

To determine the effect of transcription factors on transcriptional activity reporter plasmids were co-transfected with pcDNA3 constructs (section 2.7.4). In these experiments it was not possible to include the pRL-TK plasmid in the co-transfection experiments as the *Renilla* luciferase activity has previously been noted to be adversely affected by pcDNA3-based plasmids (Smith, Zhu et al. 2004). This 'promoter crosstalk' has been attributed to trans effects between promoters on cotransfected plasmids (Farr and Roman 1992). In these experiments variability in transfection efficiency between plates was mitigated by including control transfections with the empty vector on each plate, and results expressed as mean activity relative to that of the empty vector (\pm standard deviations).

2.12 Measurement of luciferase activity

Lysates from transfection experiments were thawed, centrifuged at 13,000 rpm for 3min and the supernatant removed. Firefly and *Renilla* luciferase activity in lysates was measured using the Dual-Luciferase™ Reporter Assay System (Promega). Luminescence was measured in a Wallac Victor 2 1420 Multilabel luminometer (Perkin-Elmer).

2.13 Statistics

Results were analysed using GraphPad Prism v5 (GraphPad Software, California, USA). Statistical significance was determined using an unpaired t -test ($p < 0.05$ indicated by *).

CHAPTER 3

DESMOSOMAL CADHERIN EXPRESSION IN COLORECTAL CANCER

3.1 Introduction

Although desmosomes are found in all epithelial tissues, their expression patterns vary. Desmocollin 2 (DSC2) and desmoglein 2 (DSG2) are common to all desmosome-forming tissues and in simple epithelia, such as the colonic mucosa, these are the only desmosomal cadherins expressed (Nuber, Schafer et al. 1995). They are constitutive components of desmosomes and both are necessary for normal desmosome function (Tselepis, Chidgey et al. 1998). Loss of either DSC2 or DSG2 in a tissue expressing only these desmosomal cadherins would be expected to result in altered desmosomal adhesion.

As part of a concurrent study, immunohistochemical analysis of a series of colorectal tumours demonstrated alterations in the patterns of desmosomal cadherin expression. Sixteen tumours were examined, of which 8 showed a reduction in DSC2 expression, as well as a re-localisation of the protein from the membrane to the cytoplasm. In addition, all 16 specimens were found to exhibit de novo expression of DSC1 protein and 7/16 exhibited de novo DSC3 protein expression.

The initial aim of the experiments described in this chapter was to confirm these findings using western blotting. Experiments were then carried out to determine whether changes in desmocollin expression were occurring at a transcriptional level using real-time RT-PCR. Desmoglein expression was then examined at the mRNA level to determine whether changes in desmoglein expression patterns occur in colorectal cancer specimens.

3.2 DSC2 protein expression in colorectal cancer

Protein was extracted from matched specimens of histologically normal tissue and tumour material from 16 patients with colorectal cancer (described in Table 2.1). The matched samples of normal and tumour protein were then examined by western blotting. Tumour samples are often heterogeneous in nature with epithelial tissue contaminated by stromal tissue. To ensure equal loading of epithelial tissue both DSC2 and cytokeratin 8 levels were measured in samples by western blotting. On visual inspection of the western blots, it was apparent that DSC2 protein expression was reduced in 11/16 tumour samples (Fig 3.1). Levels of DSC2 appeared to be unchanged in the remaining 5 samples. Image J software was used to quantify the intensity of the bands on western blots. This semi-quantitative analysis confirmed that the majority of tumours (14/16) showed a reduction in DSC2 protein expression whereas 2/16 exhibited increased DSC2 protein expression (Fig 3.2). A correlation of DSC2 expression levels with clinicopathological variables (patient age, tumour stage, tumour site) was sought. However, due to the small numbers in each group, it was not possible to draw any

meaningful conclusions. These results should therefore be viewed as an indicator to direct future investigations.

The aim of the experiments described above was to compare DSC2 protein levels in histologically normal tissue with DSC2 protein levels in matched specimens of tumour material. In addition, for completeness sake, the overall levels of DSC2 in the 16 normal samples were compared with overall levels of DSC2 in the 16 tumour specimens (after all of the samples had been normalised against the internal control, cytokeratin 8). This analysis showed the mean tumour DSC2 expression was significantly less (61.5%, $p=0.0072$) in the tumour samples than that in matched normal tissue (Fig 3.3).

Fig 3.1 DSC2 protein expression in matched normal and tumour samples

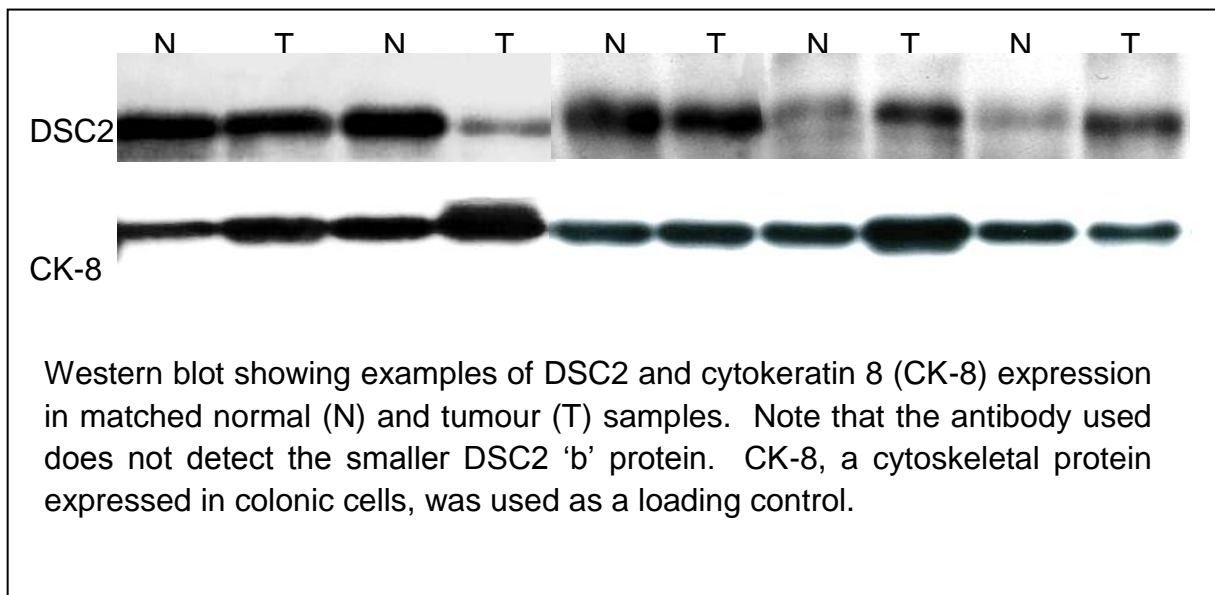


Fig 3.2 Semi-quantitative analysis of DSC2 protein expression in matched normal and tumour samples

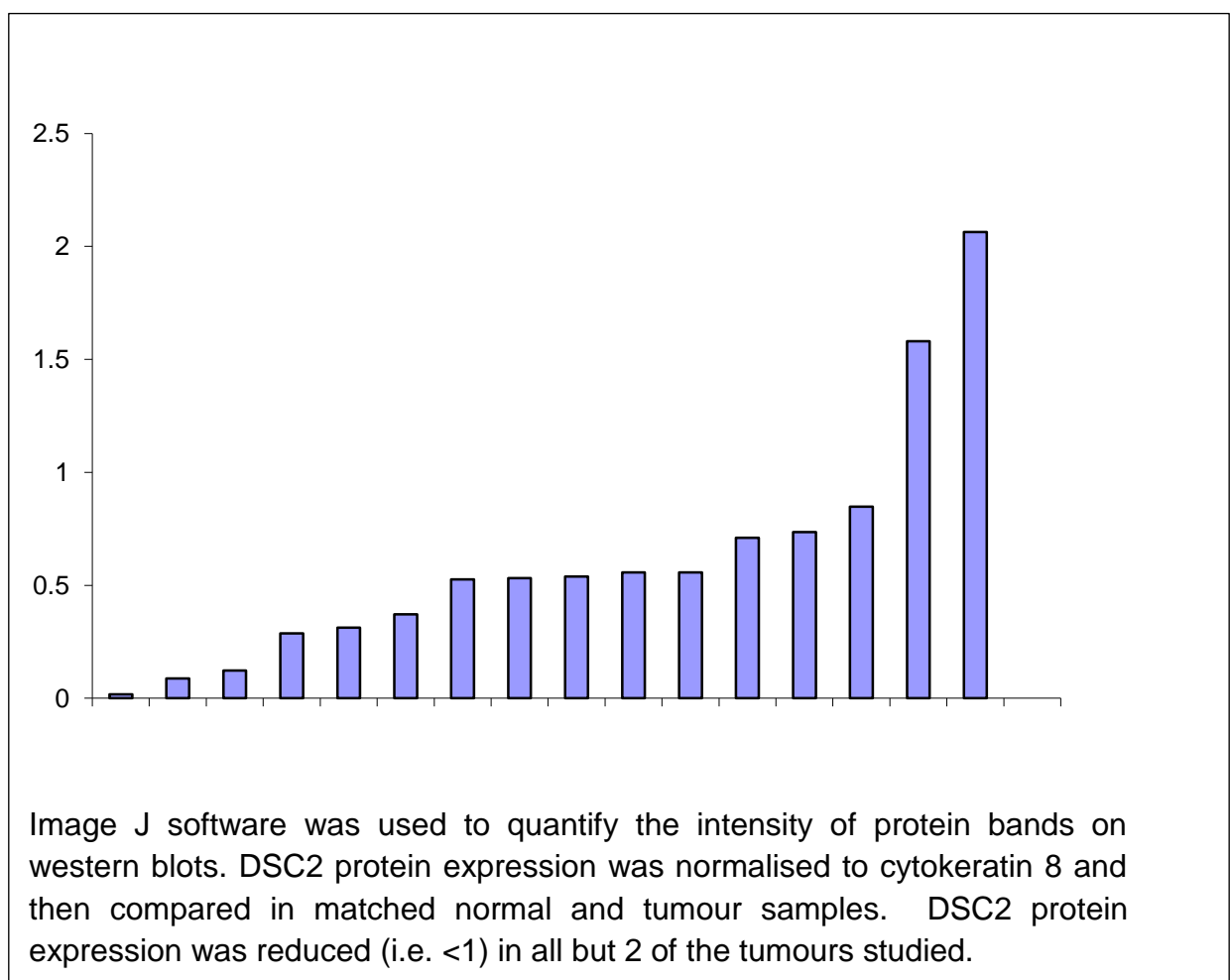


Fig 3.3 Overall levels of DSC2 protein in normal samples as compared to that in tumour samples

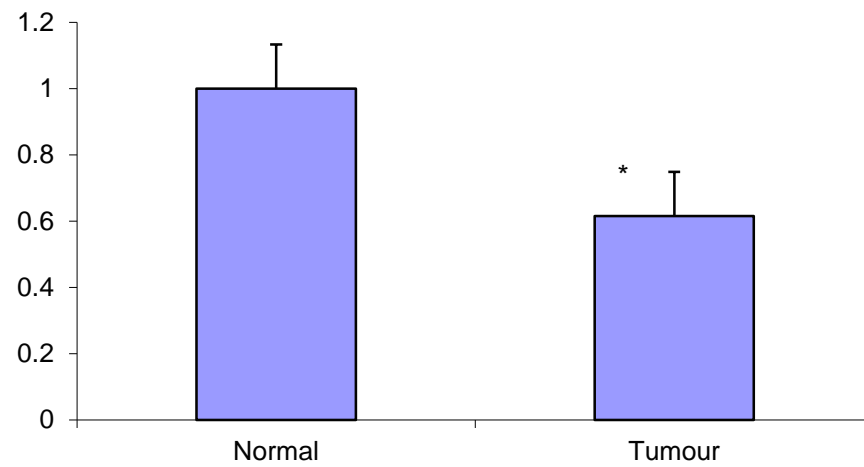


Image J software was used to quantify the intensity of protein bands on western blots. DSC2 protein expression was normalised to cytokeratin 8 and the overall expression in the 16 normal samples was compared to the overall expression in the 16 tumour samples. Overall expression in the normal samples was arbitrarily set at 1. The difference between the two sets of values was compared by unpaired t-test ($p=0.0072$).

3.3 DSC1 and DSC3 protein expression in colorectal cancer

As part of the concurrent study de novo DSC1 and DSC3 expression was detected in colorectal tumours by immunohistochemistry. Hence a series of western blots were performed to confirm these findings. DSC1 and DSC3 expression was measured in seven tumour samples and compared to that of normal colonic tissue taken from a patient undergoing surgery for a non-cancerous condition. As expected, no DSC1 or DSC3 protein was seen in the normal colonic tissue. Protein bands of the correct molecular weight were identified in 4/7 tumour samples investigated for DSC1 protein and 3/7 for DSC3 protein (Fig 3.4). Interestingly, expression of DSC1 and DSC3 was seen in samples where DSC2 protein expression was lost but also when it was maintained, suggesting that altered DSC2 expression is not necessarily the event that triggers expression of DSC1 and DSC3 (Fig 3.5).

Fig 3.4 Western blots for DSC1 and DSC3 in colorectal tumour samples

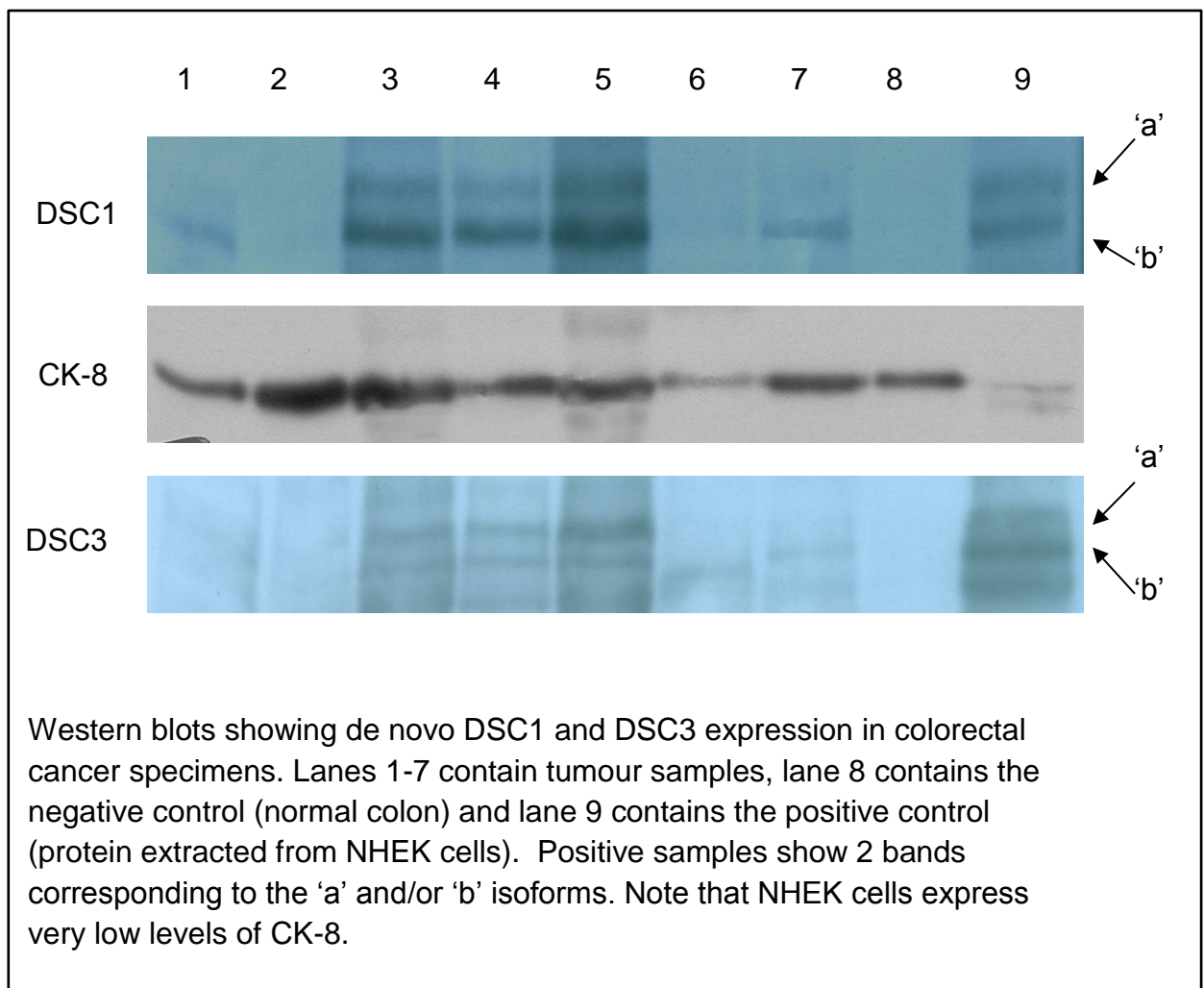
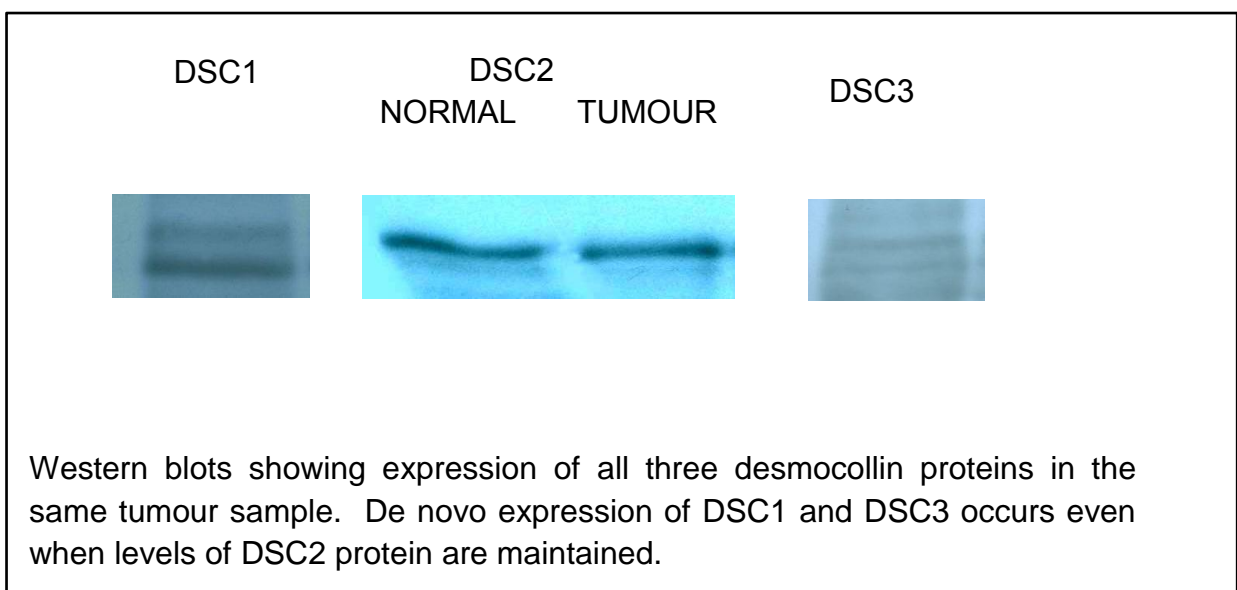


Fig 3.5 Western blots for tumour sample 20



3.4 *DSC2* transcription in colorectal cancer

Having determined that *DSC2* protein expression is decreased in colorectal cancer, the next stage of the investigation was to identify possible mechanisms by which this occurs. Specimens from 7 tumours with matched normal tissue were examined by real-time PCR to study *DSC2* mRNA expression. One sample showed increased *DSC2* expression (a 32-fold increase). However, the expression level in the majority (6/7) of samples was essentially unchanged (i.e. <2-fold change). Hence the loss of *DSC2* protein that is observed in tumour specimens by western blotting (Fig 3.1) does not appear to be due to a reduction of *DSC2* mRNA.

Again, for completeness sake, overall levels of *DSC2* mRNA in the 7 normal samples were compared with those found in the 7 tumour samples (after all of the samples had been normalised against the internal control, cytokeratin 8). There was no significant difference between overall *DSC2* mRNA levels in the normal tissue samples as compared to overall *DSC2* mRNA levels in the tumour samples (Table 3.1).

Fig 3.6 Desmocollin mRNA expression patterns in matched specimens of histologically normal tissue and tumour material

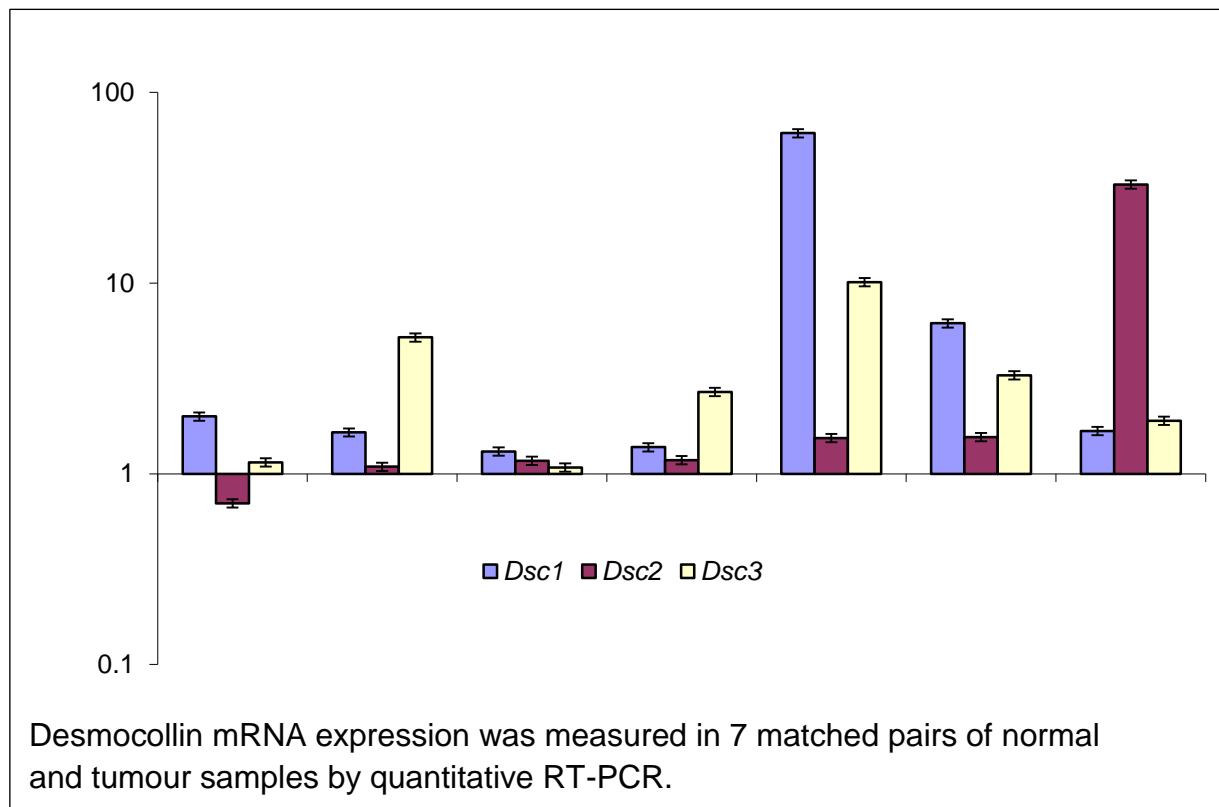


Table 3.1 Desmocollin real-time PCR data

	Normal	Tumour	P value unpaired t test
<i>DSC2</i>	19.32 (\pm 3.59)	18.49 (\pm 2.43)	0.49
CK-8	17.95 (\pm 1.82)	18.04 (\pm 1.4)	
Δ Ct	1.36 (\pm 2.86)	0.46 (\pm 1.77)	
<i>DSC1</i>	29.58 (\pm 2.28)	28.1 (\pm 1.6)	0.26
CK-8	18.07 (\pm 1.56)	18.23 (\pm 1.72)	
Δ Ct	11.5 (\pm 3.3)	9.89 (\pm 1.73)	
<i>DSC3</i>	29.01 (\pm 5.7)	27.3 (\pm 4.6)	0.32
CK-8	18.56(\pm 3.01)	18.4 (\pm 2.5)	
Δ Ct	10.5 (\pm 5.83)	8.9 (\pm 5.5)	

Table shows mean Ct values (\pm standard deviation) for 7 normal controls and the 7 tumour specimens for *DSC1*, *DSC2* and *DSC3*. Mean Ct values were also determined for internal control CK-8 and subtracted from the mean Ct values for each DSC under investigation to give Δ Ct value. The Δ Ct value for normal and tumour samples was compared by t-test.

3.5 *DSC1* and *DSC3* transcription in colorectal cancer

Having detected the *DSC1* and *DSC3* protein by immunochemistry and western blotting, further evidence to support this observation was sought by investigating *DSC1* and *DSC3* mRNA expression. Seven matched samples were evaluated for *DSC1* expression by real-time PCR (Fig 3.6). *DSC1* mRNA expression was upregulated in 3/7 tumour samples and unchanged in the remaining four samples (i.e. there was a less than 2-fold difference between the normal and tumour tissue). A correlation was found between *DSC1* message levels and protein expression. For example, the sample with the maximum increase at the mRNA level also expressed high levels of *DSC1* protein. No *DSC1* protein was detected in samples with <2-fold increase in *DSC1* transcript.

Matched samples from seven colorectal cancer patients were investigated for *DSC3* mRNA expression (Fig 3.6). *DSC3* mRNA expression was upregulated in 4/7 tumour samples and unchanged in the remaining three when compared to the matched normal tissue. Samples containing upregulated *DSC3* transcript also contained *DSC3* protein by western blotting. No *DSC3* protein was found without a corresponding increase in mRNA levels.

Although it is clear that de novo *DSC1* and *DSC3* expression occurs in some tumour samples, both at the protein and mRNA levels, there was no statistically significant difference between overall *DSC1* and *DSC3* mRNA

levels in the tumour samples and overall mRNA levels in the normal control samples (Table 3.1). This probably reflects variations in expression levels between individual patients, coupled with the small number of specimens examined, and shows the methodological advantage of comparing expression in matched specimens from the same patient (and so eliminating inter-individual variations).

3.6 Desmoglein expression in colorectal cancer specimens

At the time of this study, it was not possible to assess desmoglein protein levels in colorectal cancer samples because of a lack of desmoglein isoform-specific antibodies. However, having investigated *DSC1-3* mRNA expression, desmoglein expression was also examined by quantitative RT-PCR. Real-time PCR for *DSG1-4* was performed in six matched normal- tumour pairs. There was little, if any change in the levels of *DSG2* message in any of the matched normal-tumour paired samples investigated. However, all of the tumour samples examined did show de novo expression of *DSG1*, *DSG3* and *DSG4*, none of which are normally expressed by colonic mucosa (Fig 3.7). In all of the samples examined, de novo expression of desmoglein 1, 3 and 4 mRNA was more dramatic than that of desmocollins 1 and 3 mRNA. High levels of de novo expression of desmoglein mRNA were not necessarily mirrored by high de novo expression of *DSC1* and *DSC3* mRNA. Thus, the sample showing the greatest 'inappropriate' desmoglein response, showed little de novo expression of *DSC1* and *DSC3* mRNA (Fig 3.8).

A comparison of mean levels of mRNA in normal samples versus mean levels of mRNA in tumour samples was also performed. No significant decrease in *DSG2* mRNA levels was obtained by this method. Similarly no significant increase in *DSG1* mRNA levels was found. However, significant increases in *DSG3* and *DSG4* mRNA levels in tumour samples were observed ($p < 0.05$, unpaired t test, see Table 3.2).

Fig 3.7 Desmoglein mRNA expression patterns in matched specimens of histologically normal tissue and tumour material

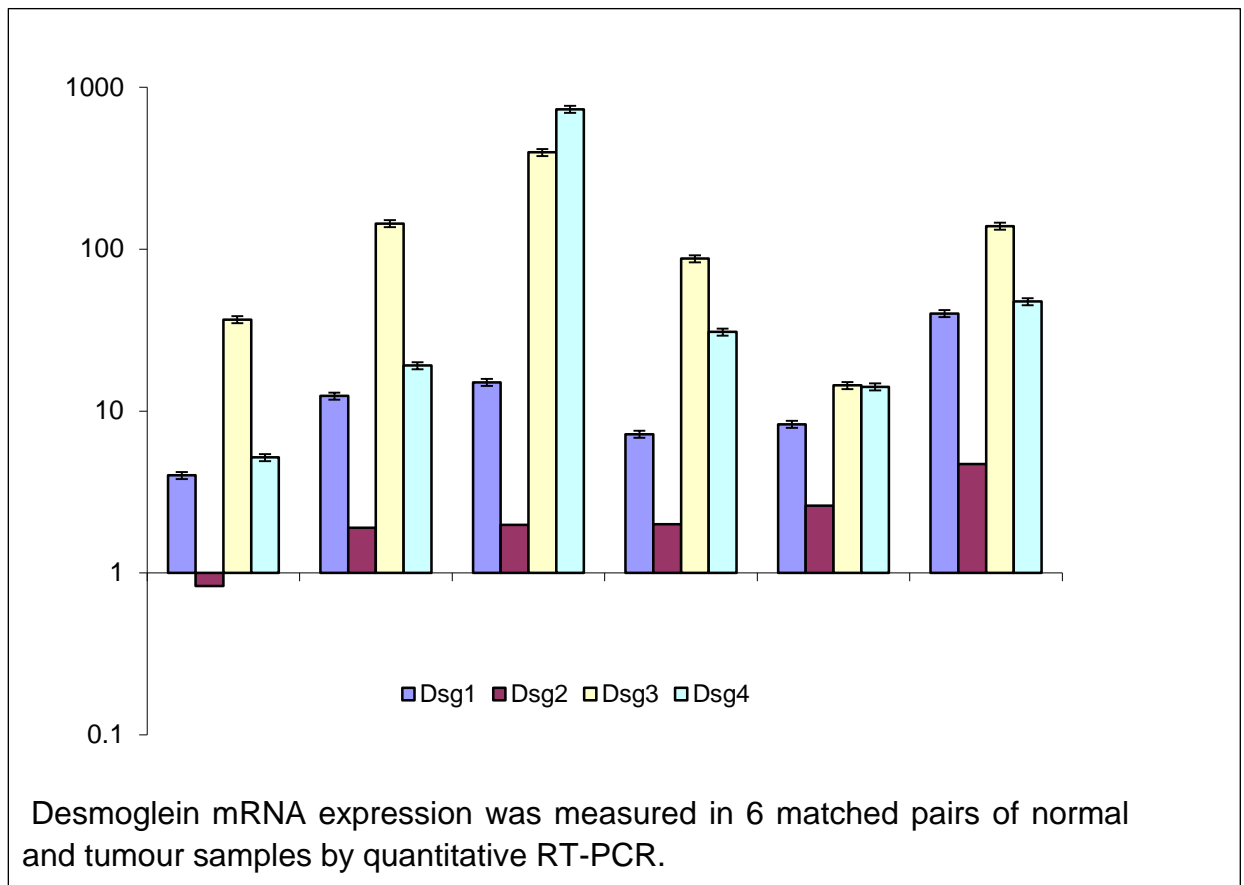


Fig 3.8 Desmocollin and desmoglein mRNA expression patterns in matched specimens of histologically normal tissue and tumour material

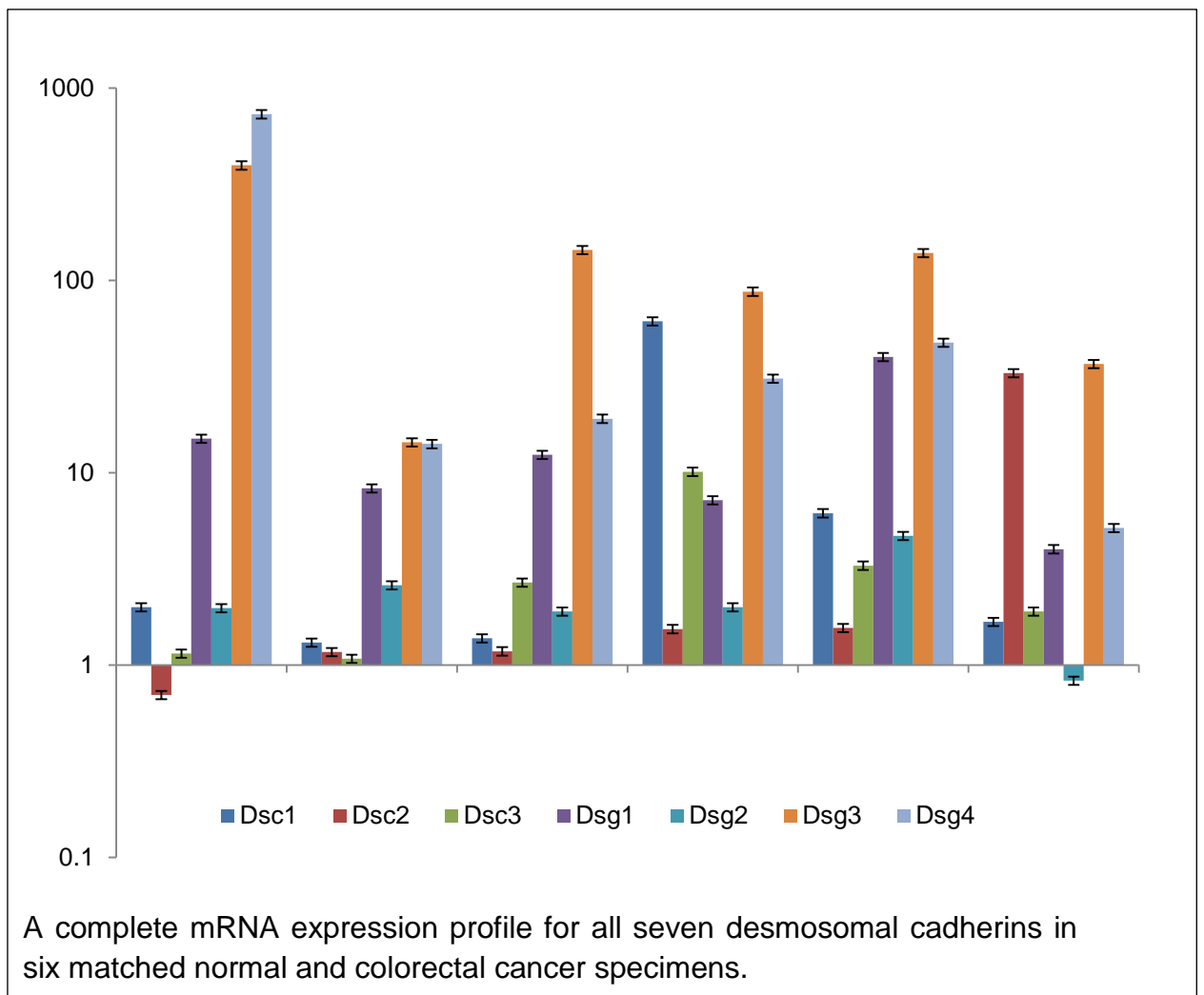


Table 3.2 Desmoglein real-time PCR data

	Normal	Tumour	P value unpaired t test
<i>DSG1</i>	31.9 (± 2.45)	30.5 (±3.43)	0.45
CK-8	18.12 (± 1.72)	18.5 (± 1.9)	
Δ Ct	13.8 (±2.93)	12 (±4.84)	
<i>DSG2</i>	17.99 (± 0.74)	17.33 (±1.19)	0.53
CK-8	18.62 (± 1.75)	18.67 (± 2.92)	
Δ Ct	-0.62 (±1.5)	-1.34 (±2.24)	
<i>DSG3</i>	30.5 (± 1.62)	24.57 (±2.71)	<0.0001
CK-8	20.37(± 1.02)	20.75 (± 1.46)	
Δ Ct	10.21 (±1.12)	3.81 (±1.74)	
<i>DSG4</i>	32.25 (± 2.68)	27.56 (± 3.3)	0.0018
CK-8	20.3(± 1.02)	20.7(± 1.47)	
Δ Ct	11.88 (±1.99)	6.8 (±2.17)	

Table shows mean Ct values (± standard deviation) for 7 normal controls and the 7 tumour specimens for *DSG1-4*. Mean Ct values were also determined for internal control CK-8 and subtracted from the mean Ct values for each DSG under investigation to give Δ Ct value. The Δ Ct value for normal and tumour samples was compared by t-test.

3.7 Discussion

This investigation has demonstrated reduced DSC2 protein expression in colorectal cancer, coupled with de novo expression of those desmocollins that are not normally expressed in normal colonic mucosa, i.e. DSC1 and DSC3. This is in keeping with the findings of an immunohistochemical study that was being carried out at the same time as the work described here. The combined data from these two studies are now published (Khan, Hardy et al. 2006). A previous study on the role of desmosomes in colorectal cancer found no evidence for loss of expression of desmocollins (Collins, Taylor et al. 1990), a result that clearly conflicts with the data presented herein. The reason for the difference between the two studies is unclear, but it could be attributed to a lack of specificity of the antibody used by Collins et al. to detect desmocollin protein. The Collins study was performed before it was known that more than one desmocollin isoform existed, and it is possible that the antibody that was used was pan-specific (i.e. able to detect all three isoforms). If this is the case, loss of DSC2 with compensatory increases in DSC1 and DSC3, would give the impression that overall desmocollin expression was unchanged. In this investigation isoform-specific antibodies were used throughout.

In colonic mucosa, DSC2 and DSG2 work together to mediate desmosomal adhesion but that is not to say that they are unable to partner other members of the desmosomal cadherin family. DSG2 has been previously shown to interact with DSC1 in a human fibrosarcoma cell line, HT1080, and mediate cell-cell adhesion (Chitaev and Troyanovsky 1997). Similarly, the co-

expression of DSC2 with DSG1 and plakoglobin in non-adhesive murine L-cells resulted in cell-aggregation (Marcozzi, Burdett et al. 1998). Thus it may be that expression of DSC1 and/or DSC3 compensates for loss of DSC2 in colorectal cancer with no overall change in desmosomal adhesion.

The loss of DSC2 protein expression does not appear to be accompanied by a reduction in *DSC2* message levels although more samples would need to be examined to definitively rule out transcriptional mechanisms. The most plausible explanation for the loss of DSC2 protein is increased turnover as a result of reduced protein synthesis or increased rates of protein degradation, perhaps as a result of loss of protein stability. As a first step in investigating this issue, protein half-life, a measure of both synthesis and degradation, could be measured in colorectal cancer cell lines by pulse chase experiments, using primary colonic cells as the control.

Although correlations were sought with clinicopathological variables, due to the small numbers of cancer specimens investigated, it was not possible to draw any significant conclusions. However, having recognised that the changes take place, future experiments could involve a greater number of patient samples as well as using samples from across the spectrum of colorectal neoplasia, from adenoma to all the different stages of invasive carcinoma. Identification of where in the sequence of events these changes

arise may help to understand the role of desmosomal cadherins in colorectal tumourigenesis.

In order to ensure that only high quality protein and RNA were used for these experiments, stringent quality control procedures were adhered to. RNA is susceptible to degradation by endogenous and exogenous RNAses, and samples were handled in the operating theatre in a standardised manner and transferred to liquid nitrogen as soon as possible. Following RNA extraction, samples were visualised on a denaturing gel to ensure the RNA was intact prior to further investigation. Prior to evaluating samples by real-time PCR, SYBR Green PCR samples were viewed on gel electrophoresis to ensure that the fluorescent signal was due to an amplicon of the correct size rather than the generation of primer dimers. This is a recognised problem associated with this technique but can be overcome with careful primer design and optimisation of the PCR. However, it is quick and relatively simple and cheaper when compared to the cost of having to design and prepare a separate hybridisation probe (e.g. Taqman) for each of the seven desmosomal cadherins.

Care must be taken when interpreting real-time PCR results that report a difference in relative activity between matched samples, as the calculated result may give the impression of more of a difference between 2 samples than there actually is. In addition, as real-time PCR is looking at relative

activity, selection of suitable controls may influence the result. A disadvantage of using matched normal tissue from the surgical specimen adjacent to the tumour is that although it may look macroscopically normal, the cells may have been subjected to a 'field change'. This phenomenon has been recognised in colorectal cancer (Chen, Hao et al. 2004) and if similar changes are seen in the tissue surrounding the tumour, an experimental technique that reports relative expression may underestimate the difference between the tumour and unaffected tissue. To mitigate this effect, future collection of specimens at time of surgery could involve taking biopsies far away from the tumour site. Alternatively, the matched normal tissue samples could be compared to a series of samples taken during a normal screening colonoscopy, to ensure that the desmosomal cadherin expression profiles were similar in both. A third way to overcome this would be to not use matched samples from the same individuals, but instead compare the panel of tumours to a series of normal samples taken from patients undergoing a normal screening colonoscopy.

Real-time PCR most commonly is utilised to assess gene expression levels relative to an internal control. The selection of an appropriate control is very important as it should be able to correct for variations in RNA in the sample, uneven loading and variations in the experimental conditions (Bustin and Mueller 2006). Rubie et al suggest that the ideal internal standard 'should be constitutively expressed by all cell types independent of experimental conditions and they should not be affected by any human disease' (Rubie,

Kempf et al. 2005). Typically housekeeping genes such as glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and β -actin, as well as 18S ribosomal RNA, are used for this purpose but studies in colorectal cancer have shown that GAPDH and 18S levels may vary between normal and tumour tissue (Rubie, Kempf et al. 2005). Instead, cytokeratin 8 was used as the internal control for the real time PCR and western blotting. It is widely expressed in many epithelial tissues and tumours and expression is maintained even in advanced colorectal cancer. It is not expressed by stromal tissue, but exhibits identical expression patterns in normal colonic mucosa, primary colonic tumours and advanced tumours and metastases (Moll, Franke et al. 1982). As a result, cytokeratin 8 can be used to correct for the variable epithelial content which may be present in malignant tissue, particularly in advanced cancer and has been successfully used as an internal control for real-time PCR in a number of studies (Bises, Kallay et al. 2004; Caldwell, Jones et al. 2004).

Similar precautions were taken with the protein analysis. Following extraction of the protein fraction using Tri-reagent, the matched normal-tumour protein samples was subjected to a Biorad assay in order to determine the concentration. Samples were then visualised using SDS-PAGE and stained with Coomassie blue to ensure equal loading. Following transfer onto the membrane, Ponceau red stain was used to check the protein bands had been successfully transferred. These techniques were used to ensure that the observed differences in protein expression were due to reduced or increased

expression rather than experimental errors. In addition the same samples underwent western blotting with cytokeratin 8. The bands were inspected visually for equal loading, as well as being analysed using Image J software.

In general, the de novo increases in desmoglein mRNA expression were much greater than that of the desmocollins. It would be interesting to ascertain whether the increases in *DSG1*, *DSG3* and *DSG4* mRNA expression are accompanied by corresponding increases in protein expression. Isoform-specific antibodies are now commercially available and so the next step would be to establish whether desmoglein 1, 3 and 4 proteins are present by western blotting, and determine by immunohistochemistry whether they co-localise with a desmocollin protein at the cell membrane or are redistributed to the cytoplasm. Desmogleins have a much greater affinity for the signalling molecule plakoglobin than the desmocollins (Chitaev, Leube et al. 1996) and it may be that de novo expression of desmogleins, or indeed desmocollins, affects cell signalling. One way to test this would be to express desmogleins or desmocollins in cultured cells and measure the effects on intracellular signal transduction pathways. It would also be interesting to determine whether expression of exogenous desmosomal cadherins in cultured cells have any effects on cell behaviour such as proliferation, migration or invasion.

CHAPTER 4

INVESTIGATION OF A FUNCTIONAL ROLE FOR DESMOSOMAL CADHERINS IN COLORECTAL TUMOURIGENESIS

4.1 Introduction

As shown in the previous chapter, desmosomal cadherin expression is altered in colorectal cancer (Khan, Hardy et al. 2006). This then raised the question as to whether these observed changes were driving the neoplastic process or were simply a by-product of it. The aim of this chapter was to examine the effect of loss of normal desmosomal cell adhesion seen in colonic mucosa and investigate impact of this on the key features of cancerous growth, namely proliferation, invasion and migration. As desmocollin 2 (DSC2) and desmoglein 2 (DSG2) are the only two desmosomal cadherins normally expressed in colonic mucosa, the hypothesis of the experiments described in this chapter was that if desmosomal cadherins play a role colorectal tumourigenesis, then loss of either of these two proteins could result in increased proliferation, invasion and migration.

To investigate the loss of DSC2 and DSG2 protein expression an antisense cDNA knockdown strategy in cultured epithelial cells was adopted. The initial plan was to use the inducible Tet-On® system (Clontech, BD Biosciences). The advantage of this system is that background, or leaky, expression of the gene of interest (or antisense cDNA in this case) in the absence of the inducer (doxycycline) is very low. Thus it is possible to carry out transfection

experiments in the absence of the inducer, safe in the knowledge that survival of cells following transfection will not be compromised by expression of antisense RNA. A human breast adenocarcinoma-derived cell line (MCF-7 Tet-On) expressing the reverse tetracycline-controlled transactivator (rtTA) was purchased for this purpose. An inducible gene expression system can be generated by stably transfecting these cells with a plasmid that expresses antisense cDNA (or any gene of interest) under the control of a suitable Tet-response element. Unfortunately, despite repeated attempts, this series of experiments failed to produce stable transfectants following transfection of the purchased MCF-7 Tet-On cells with plasmids containing DNA encoding either *DSC2* or *DSG2* antisense cDNA.

Therefore, an alternative strategy was sought. Still using an antisense RNA knockdown method, transfections were carried out using derivatives of the plasmid pBATEM2 (see chapter 2.7.1), which had previously been used in the laboratory to successfully transfect a number of different cell lines (Tselepis, Chidgey et al. 1998). The disadvantage of the pBAT system compared to the Tet-on® system, is that it is not possible to regulate expression of cloned genes with this vector. In this series of experiments, a simple epithelial cell line was transfected with pBAT constructs that constitutively expressed either antisense *DSC2* or *DSG2* RNA. Stable clones were obtained following both experiments and these were assessed for the level of knockdown achieved at the mRNA and protein level.

4.2 Selection of cell lines

In order to find a suitable cell line for the transfection experiments, four human epithelial cell lines, were screened by immunofluorescence microscopy and RT-PCR. All four cell lines investigated, CaCo2, HT-29, MCF-7 and LS174T, exhibited epithelial morphology, with punctate desmosomal staining at the cell membranes when stained with an antibody against the desmosomal protein, desmoplakin (Fig 4.1). These results suggest that all four cell lines express all of the proteins necessary for desmosome formation and that these proteins are assembled into desmosomes at the cell surface.

RT-PCR (Fig 4.2) confirmed that two of the colonic cancer cell lines (HT-29 and LS174T) and the breast cancer cell line (MCF-7) expressed only *DSC2* and *DSG2* and were therefore considered suitable for further investigation. One colonic cell line (Caco-2) expressed low levels of *DSC1*, as well as *DSC2* and *DSG2*. As the presence of *DSC1* could potentially compensate for the loss of another desmocollin, the effect of *DSC2* knockdown could be masked by this. To avoid this source of false-negative results, Caco2 cells were not considered to be suitable for these experiments. The other three cell lines appeared to be suitable candidates for further investigation.

LS174T was chosen because the adherens junction protein, E-cadherin is not expressed by these cells, due to a single base-pair deletion on chromosome 16q (Efsthathiou, Liu et al. 1999). E-cadherin has long been recognised as a

tumour suppressor and loss of E-cadherin expression has been associated with invasive features in many different human malignancies (Frixen, Behrens et al. 1991), including those of the urinary (Umbas, Schalken et al. 1992) and gastrointestinal tracts (Becker, Atkinson et al. 1994), as well as carcinoma of the breast, in particular lobular breast cancer (Berx, Cleton-Jansen et al. 1995). The intention of using a cell line already deficient in such an important cell adhesion protein was to avoid any effect on cell behaviour caused by the knockdown of either *DSC2* or *DSG2* being masked by presence of E-cadherin.

Fig 4.1 Screening of cell lines by immunofluorescence microscopy

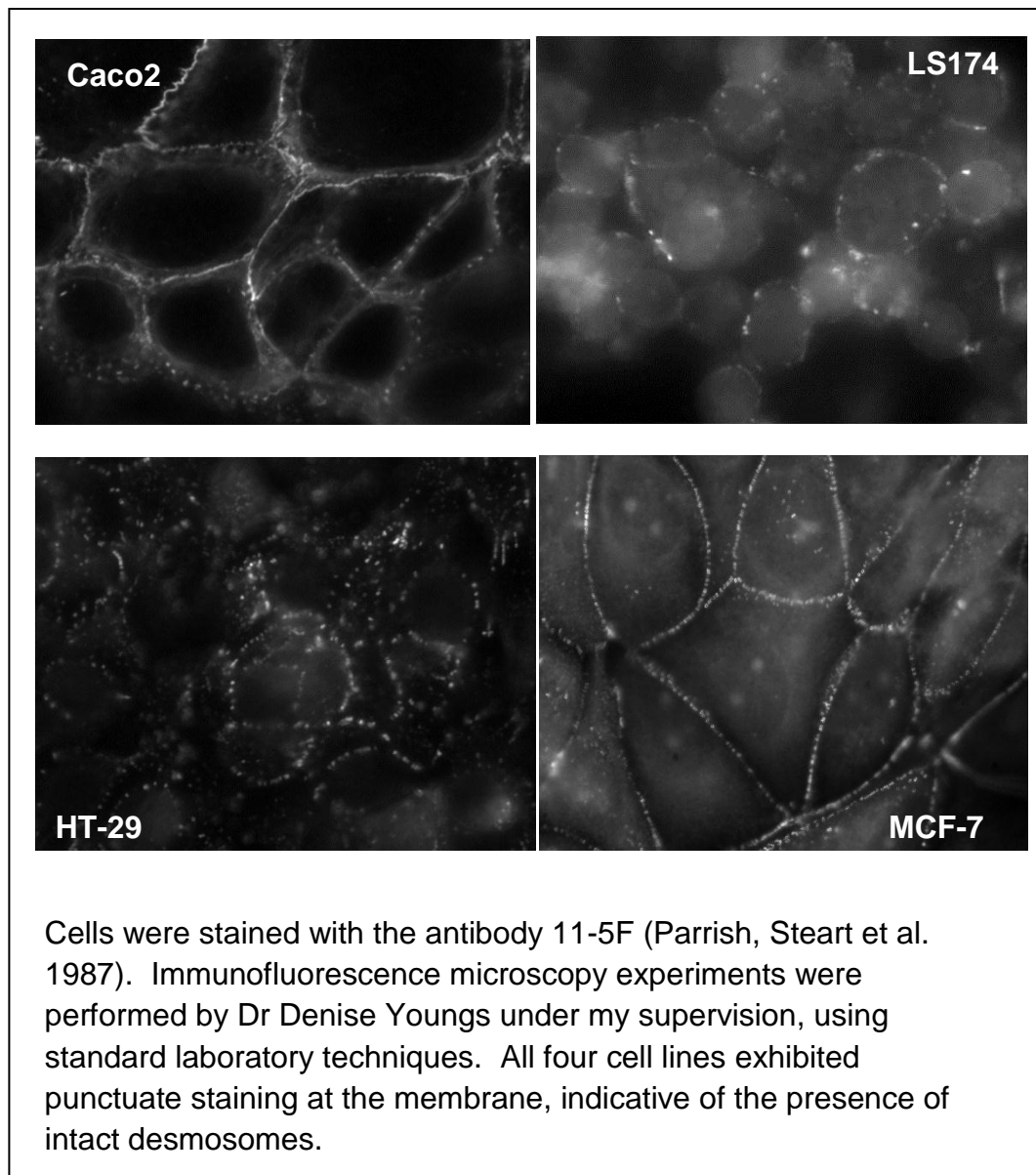
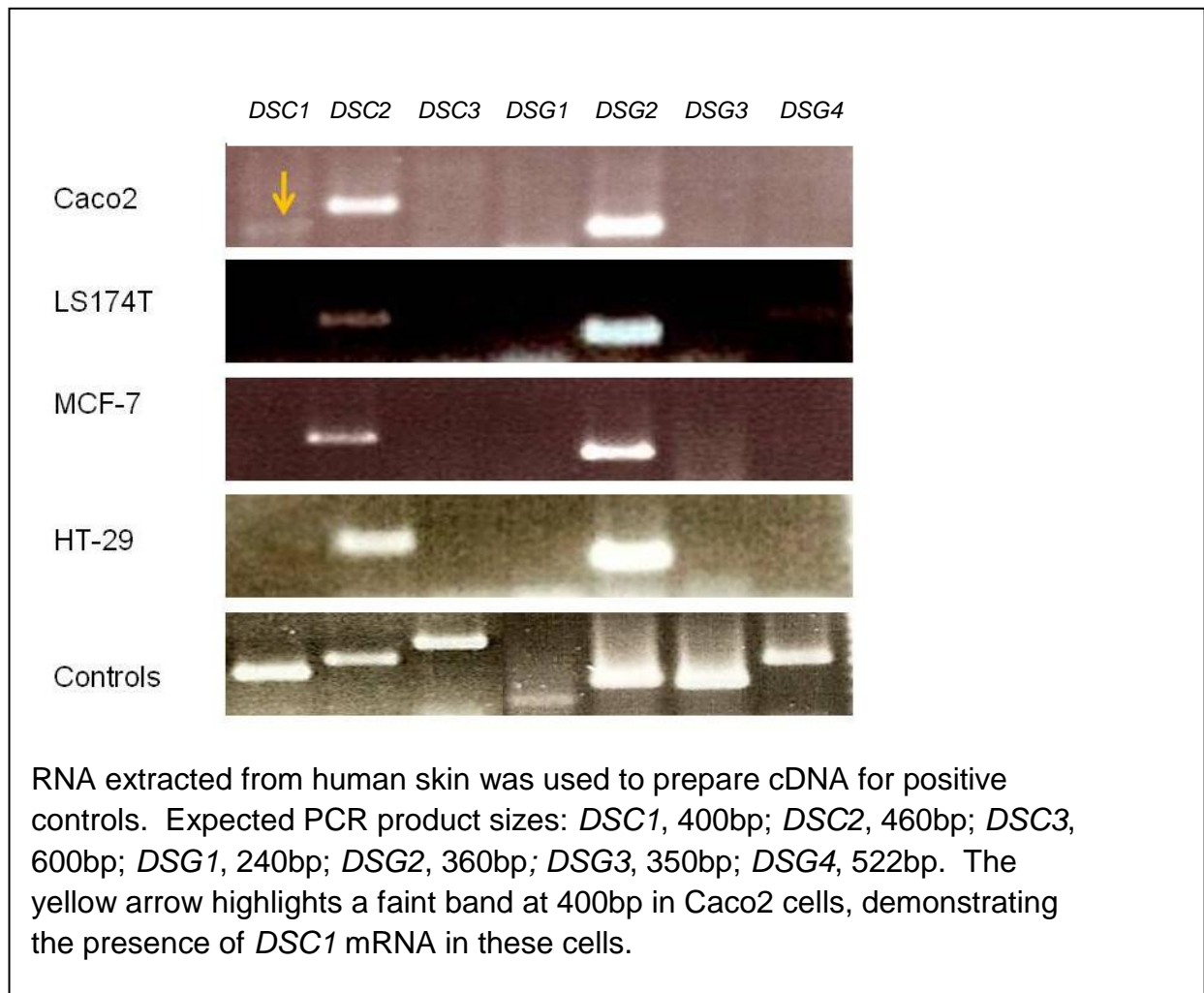


Fig 4.2 Screening of cell lines by semi-quantitative RT-PCR



4.3 Construction of *DSC2* antisense plasmids

DSC2 antisense DNA was cloned into a pBAT vector backbone, derived from the plasmid pBATEM2 (Fig 2.1), as follows: a 824bp human *DSC2* cDNA fragment was obtained by digestion of a full length human *DSC2* cDNA clone with restriction enzymes *Scal* and *Bgl*I (Fig 4.3). The fragment was blunt-ended with the Klenow fragment of DNA polymerase I and isolated by electro-elution (Fig 4.4). Similarly, a 739bp blunt-ended fragment of human *DSC2* cDNA was obtained by digestion of the full length clone with *Sph*I and *Nco*I (Fig 4.3), followed by treatment with Klenow and electro-elution (Fig 4.4). The 5.5 kb pBAT backbone was obtained by digestion of plasmid pBATEM2 with *Bgl*II and *Hind*III, followed by blunt ending with Klenow and electro-elution (Fig 4.4).

Inserts were ligated into the pBAT backbone using T4 DNA ligase. Ligation mixtures were transformed into competent XL1-Blue bacterial cells and the transformed cells plated out onto LB/ampicillin plates. Individual colonies were picked, expanded and plasmid DNA prepared using the alkaline lysis method. Plasmid DNA was screened for the presence of the insert by digestion with the restriction enzyme *Bam*HI (Fig 4.5), which linearises the pBAT vector backbone. Clones containing an insert were screened to ensure that the insert was in the correct (antisense) orientation by a combination of restriction enzyme site mapping and DNA sequencing.

Fig 4.3 Diagram showing the *DSC2* cDNA sequence and the location of the DNA fragments that were cloned into the pBAT vector backbone to produce *DSC2* antisense constructs

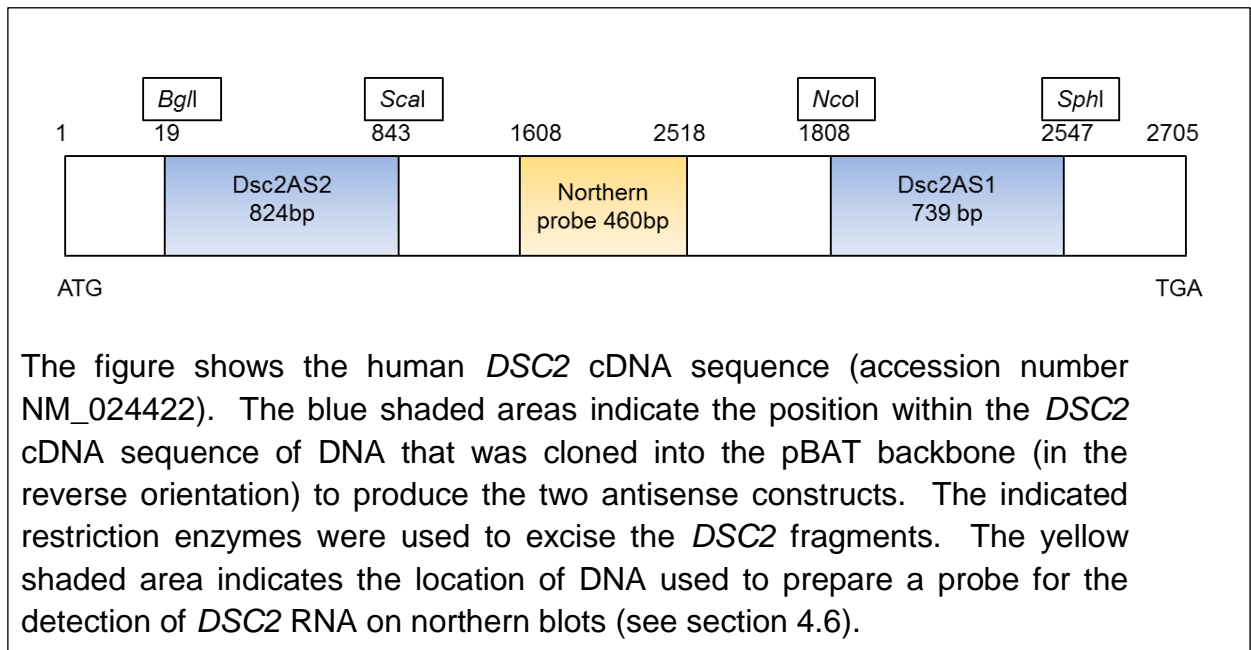


Fig 4.4 Ethidium bromide stained agarose gel showing *DSC2* and *DSG2* inserts and the pBAT vector backbone following electro-elution

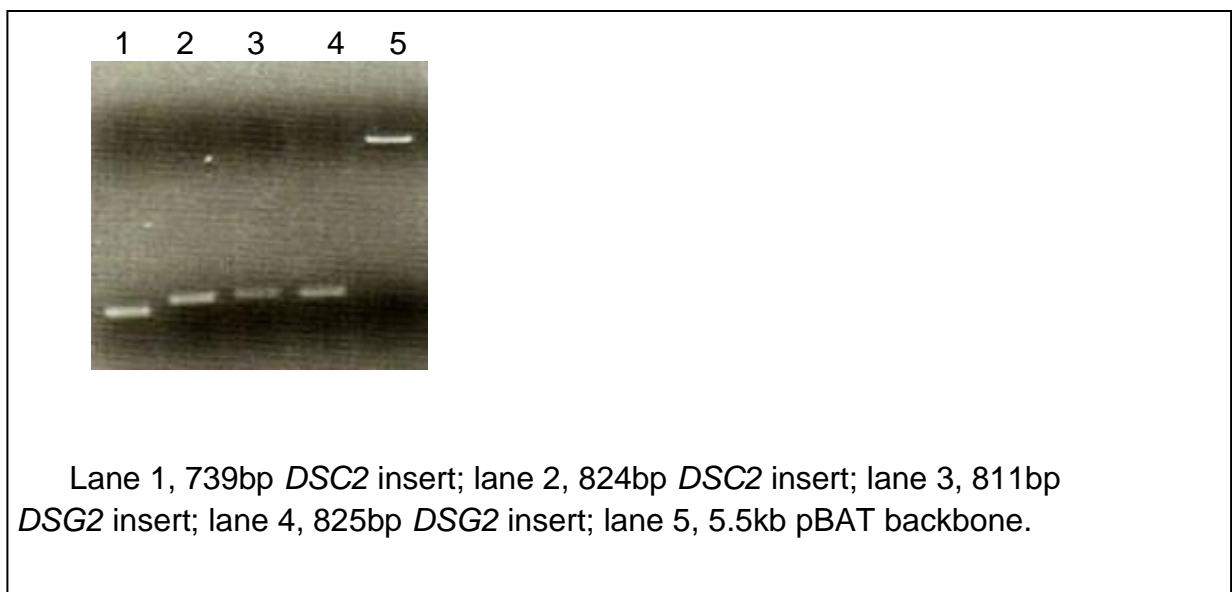
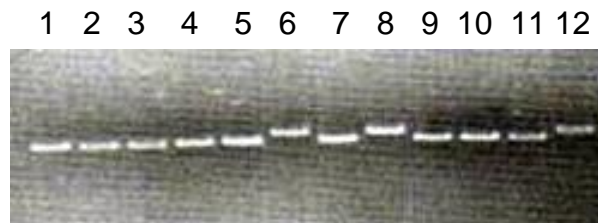


Fig 4.5 Screening of *DSC2* clones produced by ligation of the 824bp *DSC2* insert with the 5.5kb pBAT backbone.



Clones were linearised with *Bam*HI and fragments resolved by agarose gel electrophoresis. Clones C6, C8 and C12 in lanes 6, 8 and 12 respectively contain the insert, those in the remaining lanes contain vector alone.

The online software, Webcutter 2.0 (<http://www.firstmarket.com/cutter/cut2.html>) was used to identify restriction enzyme sites within the insert and backbone. This analysis revealed the presence of a *PvuII* site within the *DSC2* cDNA sequence (at position 650). A *PvuII* site is also present within the SV40 polyadenylation sequence in the pBAT backbone (Fig 4.6). Performing a digest with *PvuII* allowed the orientation of the insert to be ascertained, following ligation into the pBAT backbone, as different sized products are generated depending on orientation of the insert (Fig 4.6). Thus digestion with *PvuII* should yield a 208bp fragment if the *DSC2* cDNA fragment was inserted into the pBAT backbone in the correct (antisense) orientation and a 716bp fragment should result if the *DSC2* cDNA fragment was inserted in the incorrect (sense) orientation. Of the three clones screened (C6, C8 and C12), one (C8) contained the insert in the correct (antisense) orientation (Fig 4.7). Clone C8 named pBAT*DSC2AS2*, was sequenced to unequivocally confirm that the insert was in the antisense orientation (Fig 4.8), and used in transfection experiments (see below).

A similar restriction mapping strategy was used to establish the correct (antisense) orientation of clones obtained following ligation of the 739bp (blunt-ended) fragment of *DSC2* cDNA into the blunt-ended pBAT backbone. Again one clone containing *DSC2* in the antisense orientation was identified following restriction mapping (not shown). The clone was named pBAT*DSC2AS1* and sequenced to confirm orientation (Fig 4.8).

Fig 4.6 Digestion with *PvuII* allows orientation of the 824bp *DSC2* cDNA fragment when cloned into pBAT backbone.

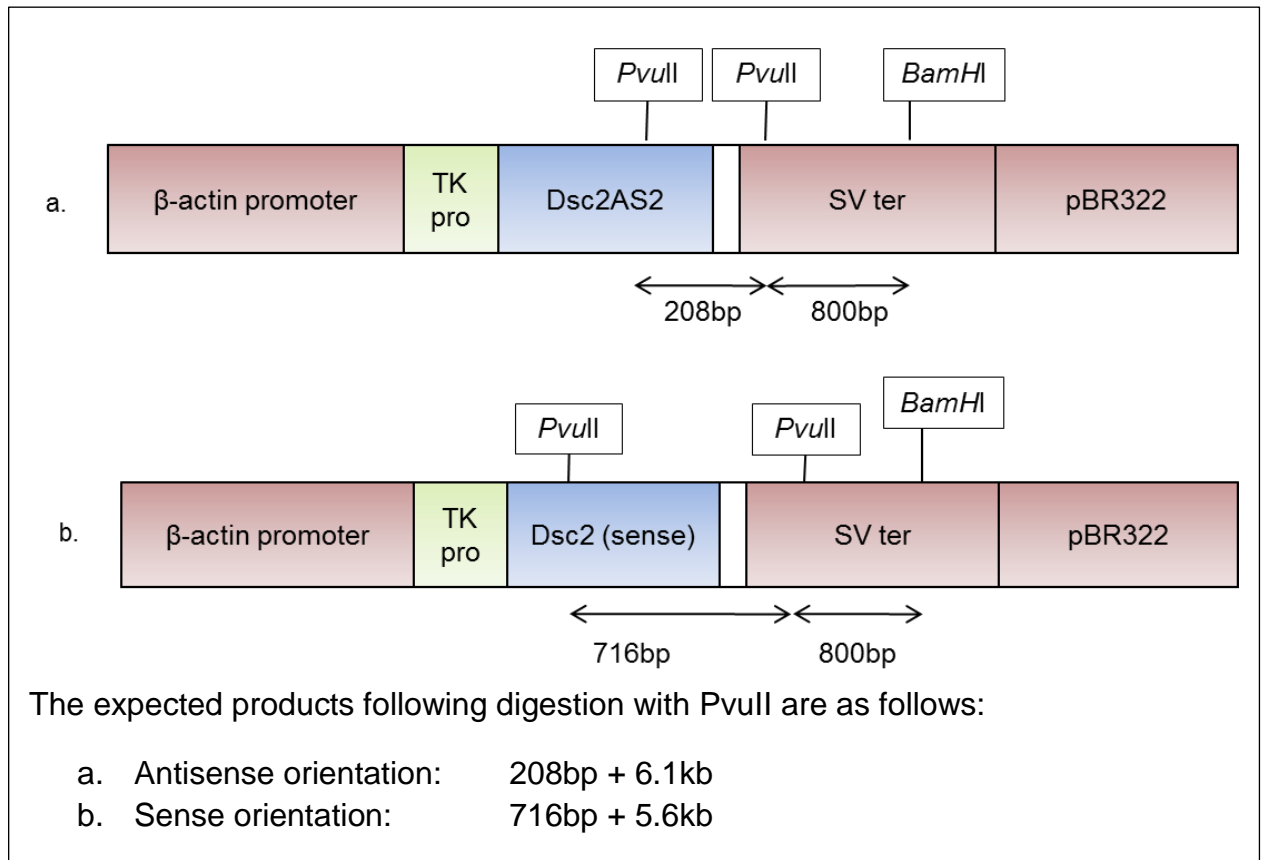
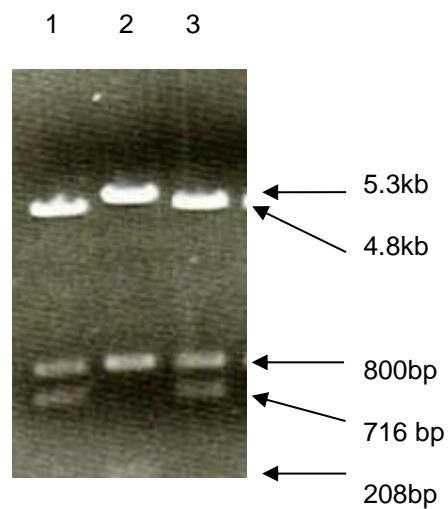


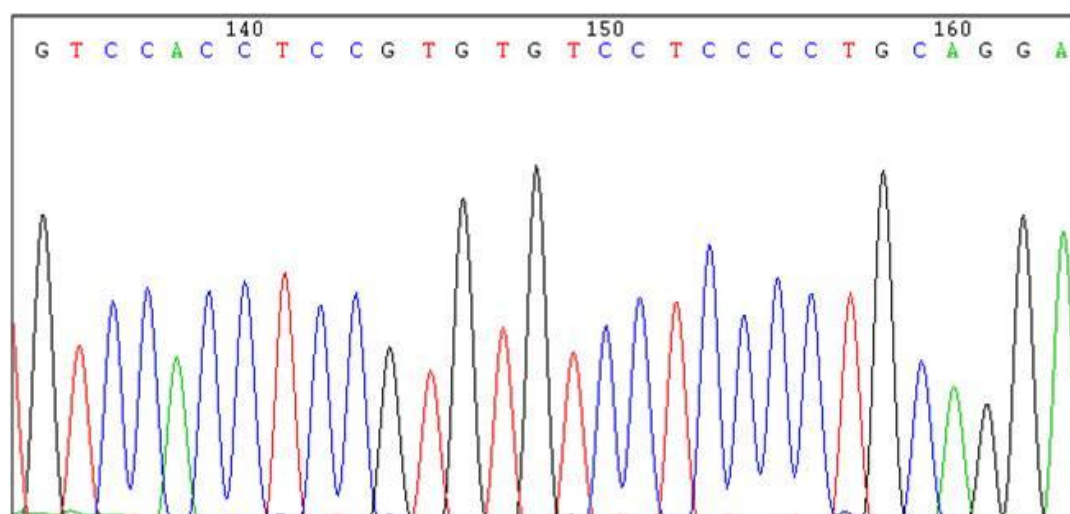
Fig 4.7 Establishing orientation of the insert produced by ligation of the 824bp *DSC2* fragment with the 5.5kb pBAT backbone



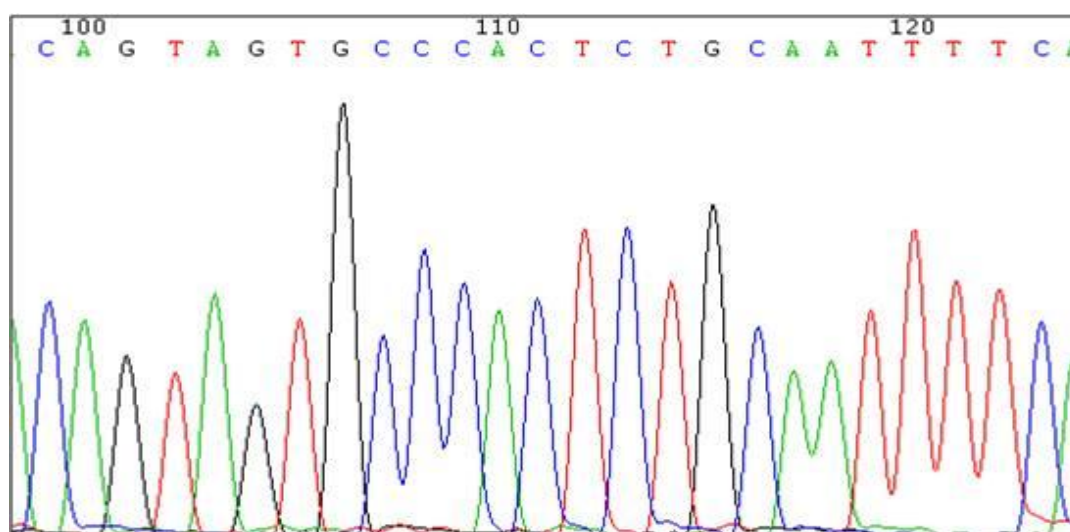
Screening of clones C6, C8 and C12 for the correct (antisense) orientation by digestion with *PvuII*. Lane 1, C6; lane 2, C8; lane 3, C12. Lane 2 contains a 208bp fragment showing that clone C8 contains the insert in the correct (antisense) orientation. Lanes 1 and 3 contain 716bp fragments showing that clones C6 and C12 contain inserts in the incorrect (sense) orientation. Note that in this experiment a double *PvuII/BamHI* digest was performed so an additional 800bp fragment is present in each lane and the size of the vector backbone is reduced accordingly.

Fig 4.8 Sequencing of pBATDSC2 AS2 and pBATDSC2 AS1 constructs

a. pBATDSC2AS2



b. pBATDSC2AS1

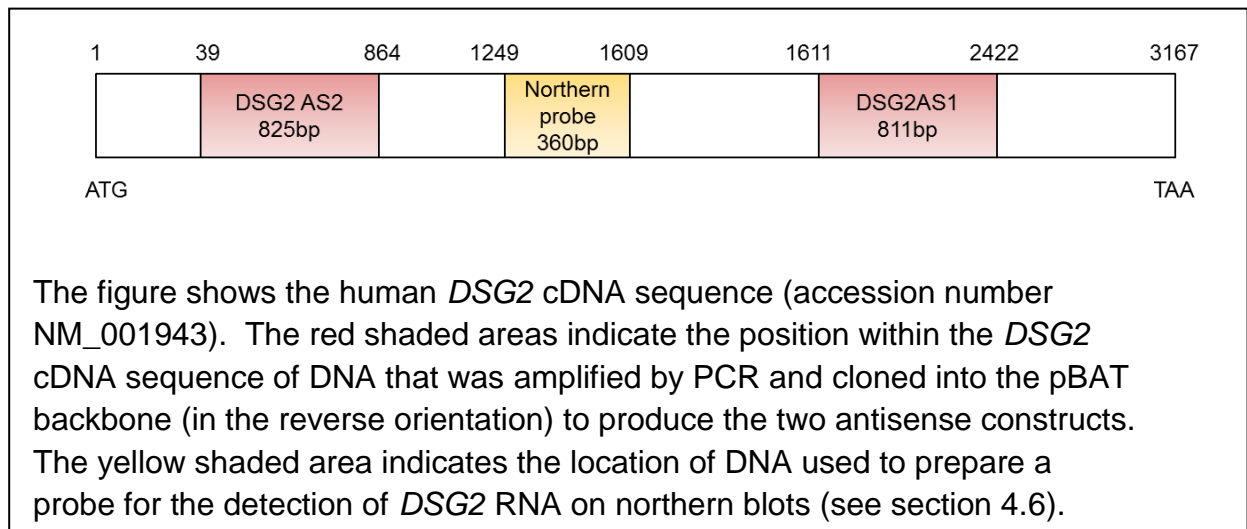


Data was obtained using a sequencing primer (TGTGGCCTCGAACACCGAG) that hybridises to the HSV TK promoter in the pBAT backbone. Results were aligned with *DSC2* sequence (NM_024422) using the Blast/Align online tool (<http://blast.ncbi.nlm.nih.gov/>). Peaks representing the 4 different nucleic acids as follows: red, thymine (T); green, adenine (A); blue, cytosine (C); black, guanine (G).

4.4 Construction of *DSG2* antisense plasmids

The following procedure was used to produce two *DSG2* antisense vectors for use in the *DSG2* knockdown experiments. Fragments of the human *DSG2* cDNA sequence (Fig 4.9) were amplified by RT-PCR using RNA extracted from human skin as starting material. An 811bp fragment was amplified using primer pair *DSG2AS2* and an 825bp fragment was amplified using primer pair *DSG2AS1* (Table 2.5). The amplified fragments were cloned into the *XcmI* cut pDK101 and sequenced to ensure that no unexpected base changes were introduced into the DNA during PCR amplification. Each set of primer pairs were designed to incorporate *SalI* and *BamHI* restriction enzyme sites. This allowed the fragments to be released from the pDK101 vector by digestion with these restriction enzymes. Following digestion the fragments were blunt ended with Klenow and isolated by electro-elution (Fig 4.4). Each fragment was then ligated into the 5.5kb blunt ended pBAT backbone. Ligation mixtures were transformed into competent XL1-Blue bacteria, and individual clones screened for the presence of the insert, and for orientation, as described in Section 4.2. Clones pBAT*DSG2AS2* and pBAT*DSG2AS1*, containing 811 and 825bp *DSG2* antisense fragments respectively, were then used for *DSG2* knockdown experiments in LS174T cells.

Fig 4.9 Diagram showing the *DSG2* cDNA sequence and the location of the DNA fragments that were cloned into the pBAT vector backbone to produce *DSG2* antisense constructs



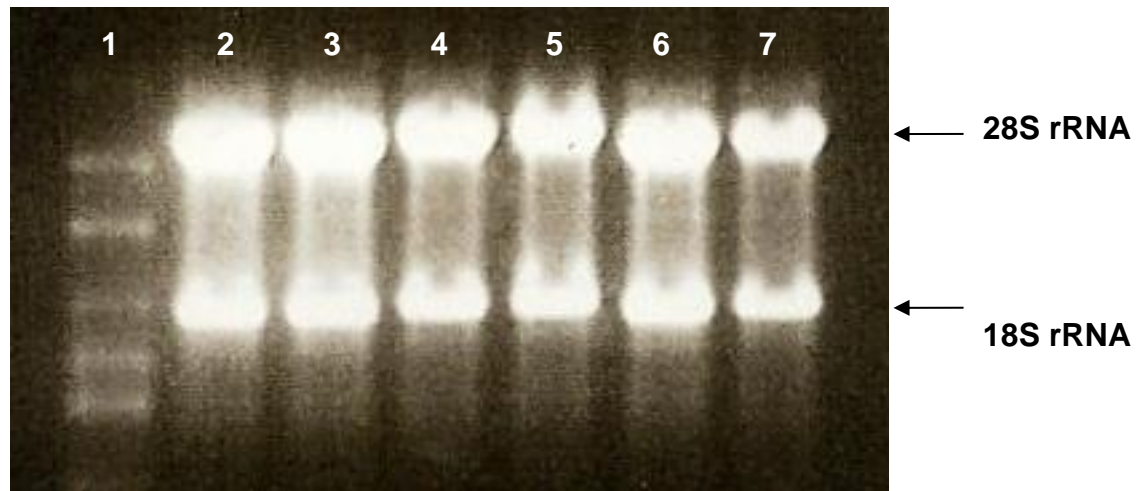
4.5 Transfection of LS174T cells with *DSG2* and *DSG2* antisense plasmids

LS174T cells were transfected as described (Section 2.11.1) with either 10µg of each of the two *DSC2* antisense plasmids (pBAT*DSC2AS2* and pBAT*DSC2AS1*), or 10µg of each of the two *DSG2* plasmids (pBAT*DSG2AS2* and pBAT*DSG2AS1*), as well as 2.5µg of the plasmid pBATneo, which confers resistance to the antibiotic G418. A third transfection was performed with 2.5µg of the plasmid pBATneo alone. Colonies were obtained in all three experiments following selection with G418. Of these, 42 antisense *DSC2*, 40 antisense *DSG2* and 8 control clones were picked and expanded.

4.6 *DSC2* and *DSG2* RNA expression in transfected cells

RNA was extracted from each of the 90 LS174T clones as described in section 2.4. These samples examined by denaturing gel electrophoresis to ensure RNA quality and equal loading (Fig 4.10). Staining with ethidium bromide and examination under UV light before and after transfer onto nitrocellulose membrane was performed to ensure transfer of RNA onto the membrane. Blots were then screened to determine whether knockdown of either *DSC2* or *DSG2* had been achieved by transfection of the cells with the antisense constructs. In order to carry out northern blotting experiments, it was necessary to first develop probes for detection of *DSC2* and *DSG2* mRNA.

Fig 4.10 Denaturing gel electrophoresis to check quality of RNA from cells transfected with antisense constructs



Ethidium bromide stained gel showing the presence of undegraded 28S and 18S ribosomal RNA. Lane1, RNA Ladder; lanes 2-4, RNA from three clones co-transfected with pBATDDSC2AS1, pBATDSC2AS2 and pBATneo; lanes 5-6, RNA from three clones co-transfected with pBATDSG2AS1, pBATDSG2AS2 and pBATneo; lane 7, RNA from one clone transfected with pBATneo alone. 10 μ g RNA was loaded per lane.

4.7 Generation of *DSC2* and *DSG2* northern probes

Probes were designed to identify *DSC2* and *DSG2* mRNA on northern blotting. Using cDNA made from LS174T RNA as starting material, a 460bp fragment of *DSC2* and a 360bp fragment of *DSG2* cDNA were amplified by PCR, using the primers listed in Table 2.3 and PCR conditions described in section 2.5.2. The locations of these fragments within the *DSC2* and *DSG2* cDNA sequences are indicated in figures 4.3 and 4.9 respectively. The PCR products were electro-eluted and then ligated with *XcmI* cut pDK101. Ligation mixtures were transformed into competent XL1-Blue cells and transformed cells grown on LB/ampicillin plates. Mini-prep DNA extraction was performed and the DNA linearised with *EcoRV* to identify colonies which had taken up the insert (Fig 4.11). A number of clones with inserts were identified. Of these, one containing a *DSC2* insert and one containing a *DSG2* insert was sequenced to confirm the identity of the insert DNA. Sequenced inserts were released from the vector backbone by digesting with enzymes *AatII* and *SacII*. The *AatII*/*SacII* fragments were then isolated by electro-elution (Fig 4.12) and labelled with radio-active ^{32}P (see section 2.5.5).

Fig 4.11 Development of northern probes: screening of clones produced by ligation of the *DSC2* and *DSG2* PCR products with pDK101

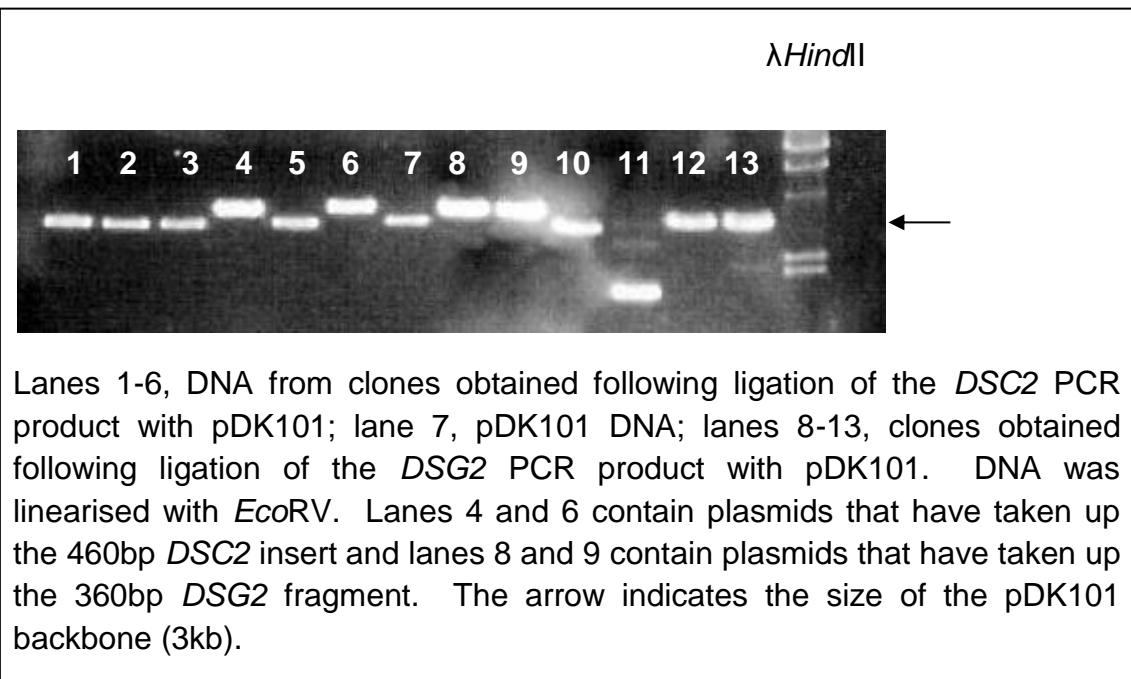
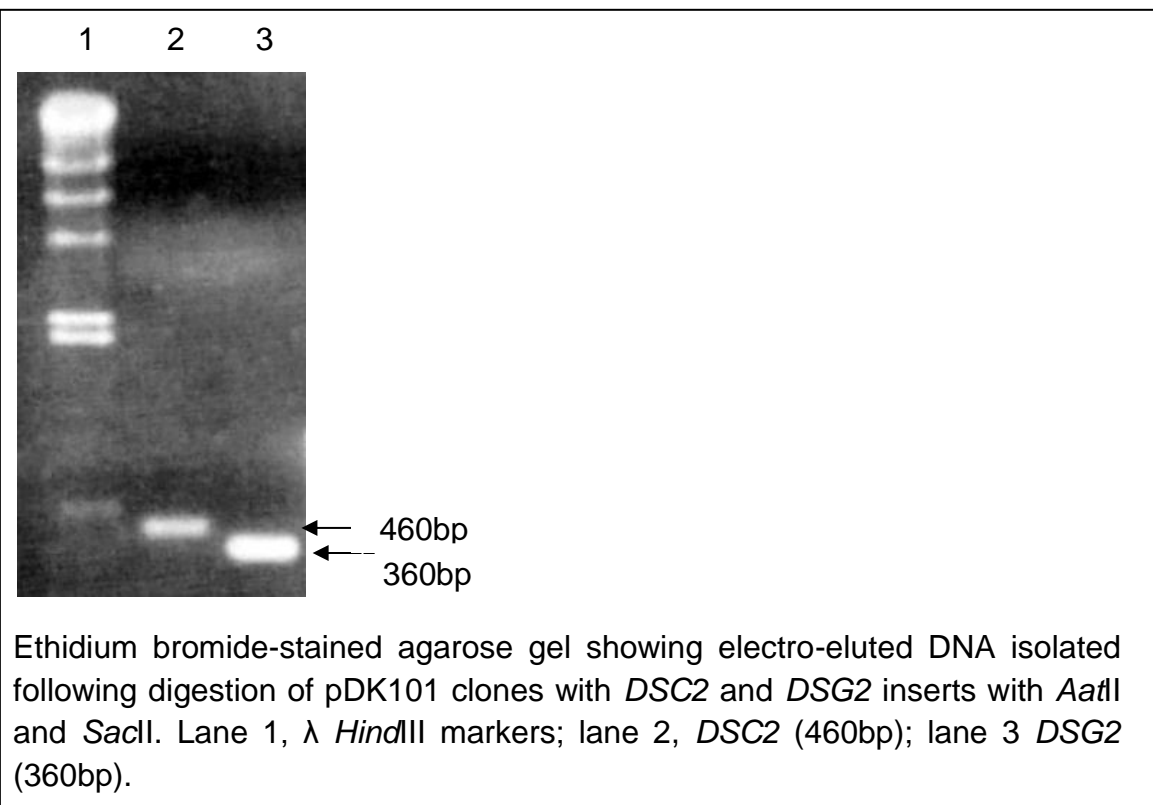


Fig 4.12 Development of northern probes: electro-eluted *DSC2* and *DSG2* cDNA prior to labelling with radioactive isotope



4.8 Assessment of *DSC2* and *DSG2* RNA knockdown by northern blotting

Once the RNA from the cells transfected with the *DSC2* or *DSG2* antisense plasmids had been transferred onto the nitrocellulose membrane and probed with the 32 -P labelled *DSC2* or *DSG2* probes respectively, the membranes were exposed to auto-radiography film for at least 1 week. Screening by northern blotting identified 10 clones in which the *DSC2* antisense plasmid appeared to have decreased *DSC2* mRNA expression (Fig 4.13). Ten clones with reduced *DSG2* mRNA expression were also identified in this manner (Fig 4.14).

Fig 4.13 Northern blot of RNA from clones transfected with antisense *DSC2* plasmids

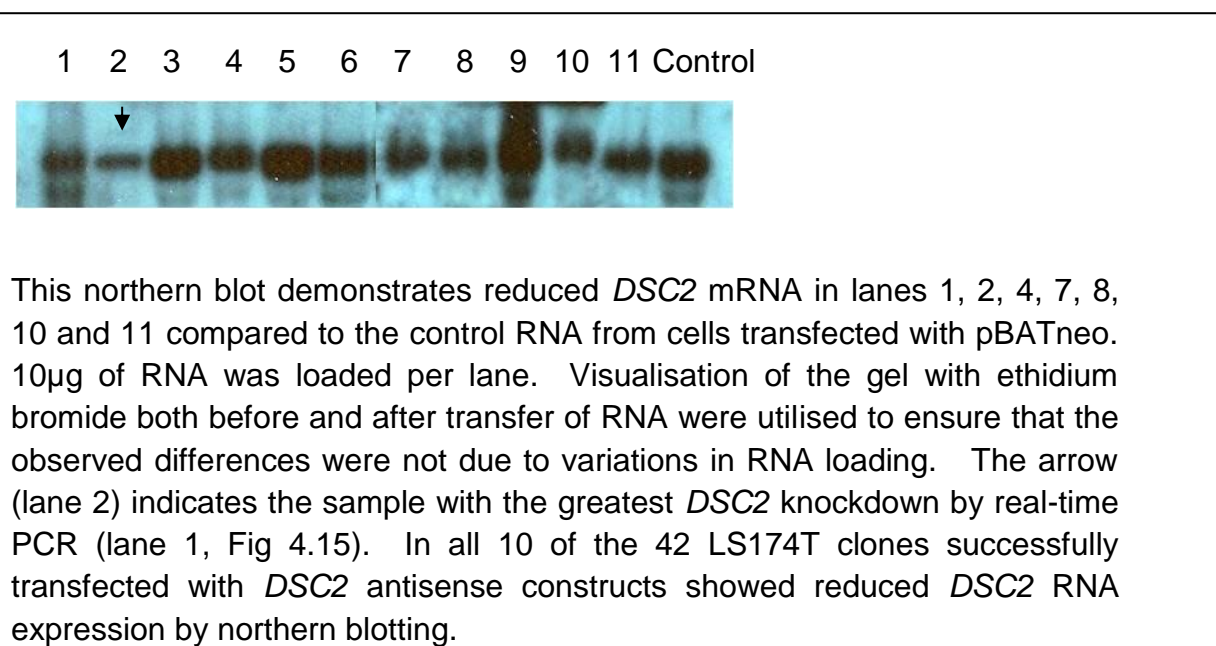
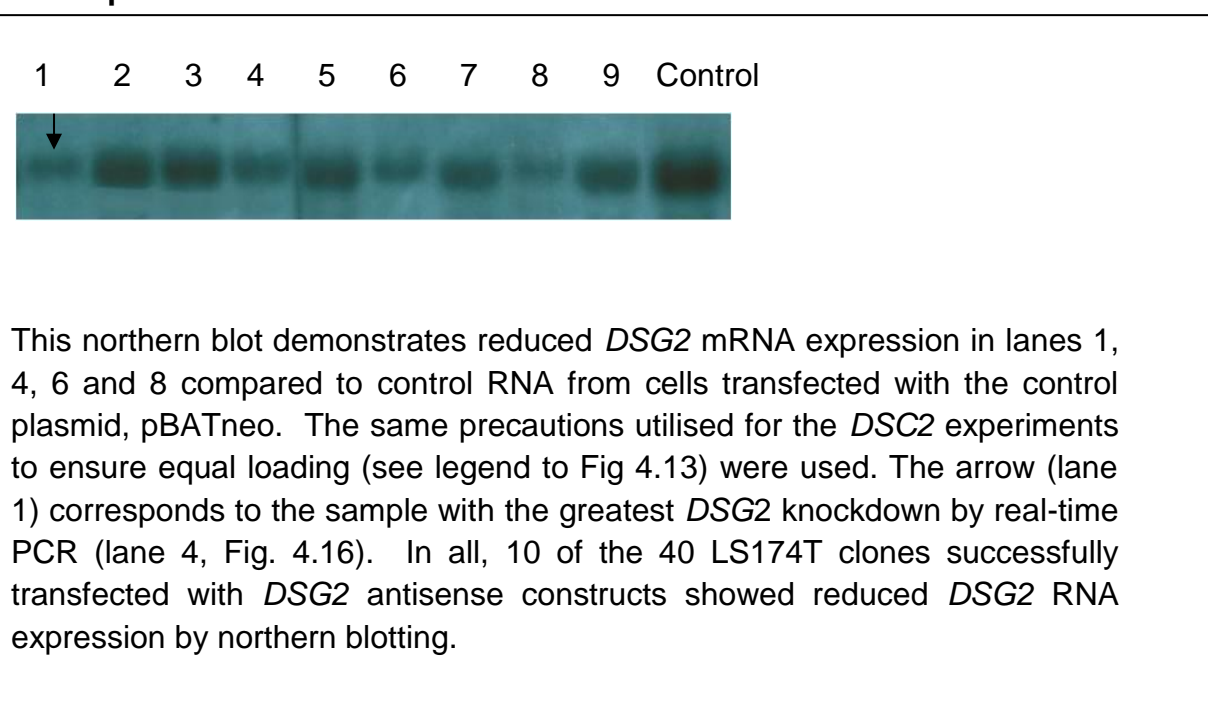


Fig 4.14 Northern blot of LS174T clones transfected with antisense *DSG2* plasmids



4.9 Assessment of *DSC2* and *DSG2* knockdown by real-time quantitative RT-PCR

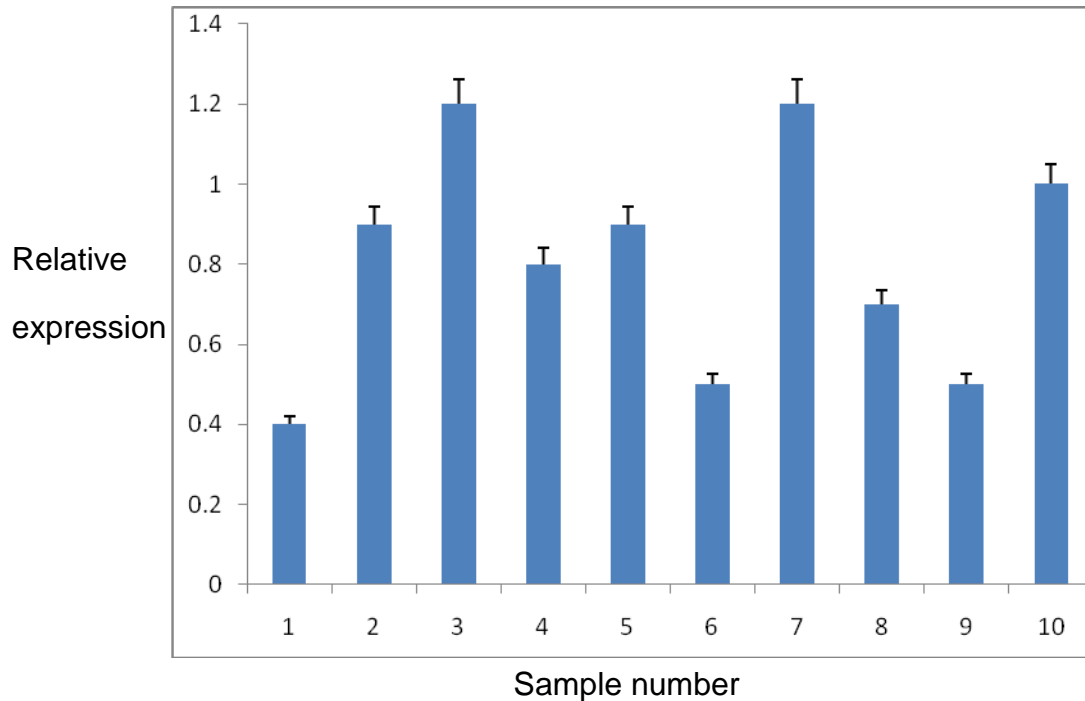
Real-time PCR was used to quantify the amount of target knockdown achieved by the expression of the *DSC2* or *DSG2* antisense plasmids. The 10 *DSC2* clones and 10 *DSG2* clones identified as showing reduced RNA expression by northern blotting were investigated by real-time PCR as described in section 2.5.1 with the primers detailed in Table 2.2. The results obtained were first normalised against cytokeratin 8, in order to correct for any variations in RNA content in the initial RT reaction. *DSC2* and *DSG2* mRNA expression in the transfected cells was then compared to the expression observed in the control transfection (with pBATneo).

Of the ten samples assessed for *DSC2* mRNA knockdown, mRNA expression was essentially unchanged in 5 (expression levels 0.8-1.2), when compared to the control. These expression levels were regarded as unchanged, as they were unlikely to result in an appreciable change in expression of the *DSC2* protein. Four of the *DSC2* knockdown samples investigated in this manner showed reduced *DSC2* mRNA expression (0.4-0.7 of control). The maximum *DSC2* knockdown achieved was 60%, i.e. *DSC2* expression was 40% of the control mRNA expression (Fig 4.15, sample 1).

The ten clones identified by northern blotting as having reduced *DSG2* RNA expression were also investigated by real-time PCR. Six of these showed unchanged mRNA expression. Reduced mRNA expression was seen in 4/10

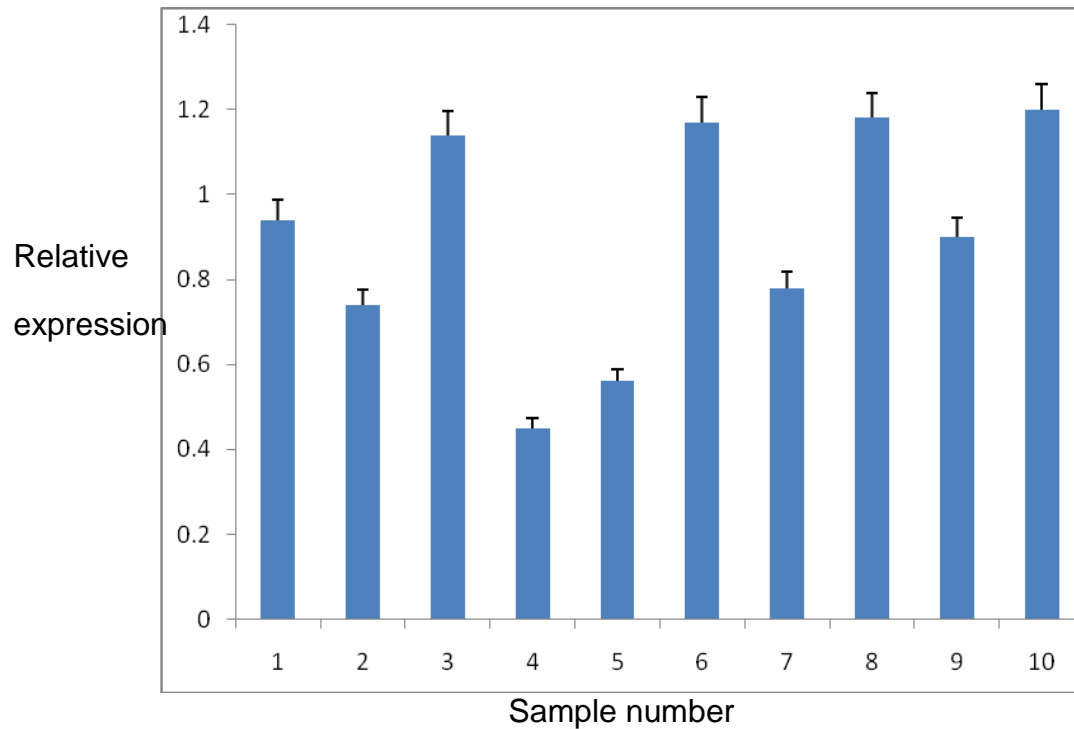
samples (0.45-0.75 of control). The maximum *DSG2* knockdown achieved was 55%, i.e. *DSG2* expression was 45% of the control mRNA expression (Fig 4.16, sample 4).

Fig 4.15 Quantification of *DSC2* knockdown in transfected LS174T cells by real- time RT-PCR



Ten clones with reduced *DSC2* mRNA expression by northern blotting were assessed by real-time PCR. All experiments were performed in triplicate with positive and negative controls. All results are expressed as a ratio of the *DSC2* expression observed with the control (LS174T cells transfected with pBATneo). The sample in lane 1 corresponds to the sample that showed the greatest *DSC2* knockdown by northern blotting (lane 2 in Fig 4.13).

Fig 4.16 Quantification of *DSG2* knockdown in transfected LS174T cells by real- time PCR

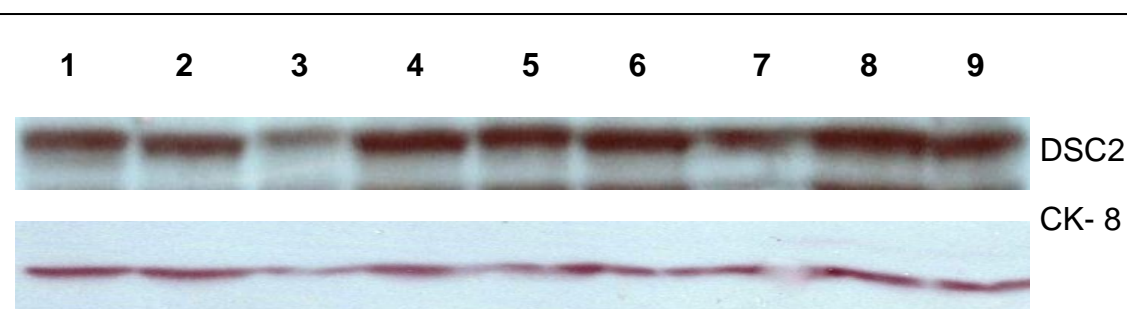


Ten *DSG2* clones with reduced *DSG2* RNA expression by northern blotting were assessed by real-time PCR. All experiments were performed in triplicate with positive and negative controls. All results are expressed as a ratio of the *DSG2* expression observed with the control (LS174T transfected with pBATneo). The sample in lane 4 corresponds to the sample that showed the greatest *DSG2* knockdown by northern blotting (lane 1 in Fig 4.14).

4.10 Protein expression in transfected LS174T cells

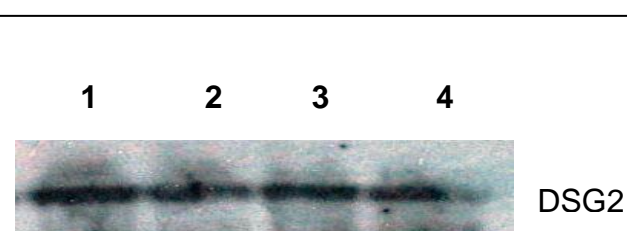
Western blotting was used to assess whether the degree of mRNA knockdown achieved in antisense transfection experiments was sufficient to alter protein expression. Protein was extracted from those 10 clones that showed reduced *DSC2* mRNA expression by northern blotting following transfection with the *DSC2* antisense constructs. Protein was resolved by SDS-PAGE and DSC2 protein detected with an anti-DSC2 antibody following western blotting onto nitrocellulose. No change was seen in DSC2 protein levels, in any of the ten clones (Fig 4.17). Similarly, those 10 clones that showed reduced expression of *DSG2* mRNA by northern blotting were investigated by western blotting with an anti-DSG2 antibody. Again, no change was seen in DSG2 protein levels (Fig.4.18).

Fig 4.17 DSC2 protein expression in LS174T clones transfected with *DSC2* antisense constructs



Protein (10 μ g) from cell lysates was resolved on a 8% SDS-polyacrylamide gel. DSC2 expression was detected using an anti-DSC2 antibody. Lanes 1-2 contain protein from control clones; lanes 3-9 contain protein from 7 DSC2 knockdown clones. This blot initially gives the impression that DSC2 protein is reduced in sample 3 and sample 7. However, the cytokeratin 8 protein expression is also reduced suggesting that the perceived differences are actually due to variations in protein loading rather than reduced DSC2 protein expression. Note that lanes 3, 4, and 5 contain protein from those clones shown by real-time PCR to have the greatest reduction in *DSC2* mRNA expression. The molecular weight of the DSC2 protein is 120kDa and that of cytokeratin 8(CK-8) is 52kDa.

Fig 4.18 DSG2 protein expression in LS174T cells transfected with *DSG2* antisense constructs



DSG2 expression was detected using an anti-DSG2 antibody. Lane 1 contains protein from a control clone; lanes 2-4 contain protein from 3 DSG2 knockdown clones. The blot was reprobed with an anti-cytokeratin 8 antibody to ensure equal loading (not shown). Molecular weight of the DSG2 protein is 160kDa.

4.11 Discussion

The outcome of this series of experiments was that although a reduction in the level of *DSC2* and *DSG2* mRNA was achieved, this was not sufficient to result in a change of protein expression. As a result, the planned investigations into the effect of loss of *DSC2* and *DSG2* adhesion proteins in epithelial tumourigenesis were not pursued. Had the antisense experiments resulted in a reduction of either *DSC2* or *DSG2* protein expression, the intention was to assess the impact of knockdown on desmosome structure (using immunofluorescence, confocal and electron microscopy) and cell phenotype (by proliferation, migration and survival assays).

The initial strategy was to use an inducible system to knockdown the *DSC2* and *DSG2* targets. One advantage of using the Tet-On® system is that the antisense plasmids would have been turned off until the inducer doxycycline was added to the culture medium. Another is that doxycycline can induce target gene expression in a dose-dependent manner. The Tet-On® system is also reversible, unlike some other inducible systems, on withdrawal of the inducer. The Tet-systems are able to regulate gene expression very closely and reduce background 'leaky' expression, which was a potential shortcoming in a previous antisense *DSC2* knockdown study, which employed a dexamethasone-inducible system (Roberts, Burdett et al. 1998). It is unclear why the system did not work in our case. The purchased MCF7 Tet-On cells grew as expected until transfected with the *DSC2* or *DSG2* antisense constructs, after which no colonies were established. The cells died before

any doxycycline had even been added to the experiment, ruling this out as a source of potential toxicity. The antisense cDNAs were later successfully transfected into LS174T cells so it is unlikely they were intrinsically lethal to mammalian cells.

Despite the successful transfection of LS174T cells with the antisense plasmids, using the non-inducible system, the observed level of *DSC2* and *DSG2* RNA knockdown did not translate to an appreciable difference in protein expression. One possible explanation for this is that the transfection was actually highly successful at achieving knockdown, but that this situation was not compatible with cell survival, thus allowing only cells with lower levels of knockdown to survive. Previous studies have shown that at least one desmocollin and one desmoglein must be present for normal desmosomal adhesion to take place (Marcozzi, Burdett et al. 1998; Tselepis, Chidgey et al. 1998), and that knockout of *Dsg2* in mice results in embryonic lethality (Eshkind, Tian et al. 2002). It is therefore possible, in simple epithelial cell line such as LS174T which only expresses one desmocollin (i.e. *DSC2*) and one desmoglein (i.e. *DSG2*), that if either of these components is lost the cell cannot survive.

Alternative strategies to the one we adopted would have been to use antisense oligonucleotides or RNA interference, in the form of siRNA. Both methods mediate gene knockdown by binding to the target RNA in a

sequence-specific manner but they achieve gene silencing by different routes. Antisense oligonucleotides usually are made up of 15-20 nucleotides and although various mechanisms have been described, the most widely adopted and often the most effective forms of antisense knockdown employ either RNase-H mediated cleavage to break down the mRNA in question or inhibition of its translation (Achenbach, Brunner et al. 2003). Although this represents a relatively simple concept, in practice, antisense oligonucleotide technology is anything but simple. One study found that only 1 in 8 antisense oligonucleotides are able to bind effectively and specifically to the target mRNA (Stein 2001). A number of reasons contribute to this, including the target sequences not being accessible due to secondary and tertiary structures formed by long RNA molecules and instability of unmodified oligodeoxynucleotides due to degradation by endogenous nucleases. In addition, low affinity of the antisense oligonucleotide for the target may require higher concentrations, leading to toxicity (Kurreck 2003). RNA interference utilises the dicer enzyme to cleave double stranded RNA molecules into shorter fragments of RNA, which bind to the target mRNA with the complimentary sequence, initiating its degradation. RNA interference is now routinely used and would be the technique of choice in any future attempts to knockdown *DSC2* or *DSG2*.

With the benefit of hindsight, a potential false negative result must also be considered. The antibody used for the detection of the DSG2 protein, 33-3D, is also capable of detecting the cytoplasmic tail of DSG1 (Vilela 1995, Kronic

1998). When the experiment was originally conceived and undertaken this did not present a problem as LS174T cells only express one desmoglein (DSG2) and the hypothesis was to examine the effects of loss of this protein. Now that there is evidence for desmosomal cadherin switching in colorectal cancer (Khan et al 2006), it is important to consider the possibility that de novo expression of DSG1 may have occurred in the knockdown cells masking any loss of DSG2 (as detected with antibody 33-3D). This could now be addressed by using a DSG1 and DSG2 specific antibodies, as well as looking for upregulation of *DSG1* at a transcriptional level.

As cadherin switching has now been recognised in both classical (Hazan, Phillips et al. 2000; Tomita, van Bokhoven et al. 2000) and desmosomal cadherins (Khan et al. 2006) any future knockdown studies, perhaps using RNA interference, should include investigation of DSC1 and 3 and DSG1 and 3 at both the protein and RNA levels. The desmosomal cadherin genes form a tight cluster on chromosome 18q (Hunt, Sahota et al. 1999), suggesting that their transcription could be regulated in a coordinated fashion. Little is known about the regulation of desmosomal cadherin gene expression and this will comprise the area of investigation for the next chapter.

CHAPTER 5

REGULATION OF DESMOSOMAL CADHERIN GENE EXPRESSION IN COLON CANCER CELLS

5.1 Introduction

Little is known about the regulation of desmosomal cadherin gene expression in colonic cells. However, the CCAAT/enhancer binding protein (C/EBP) family has previously been shown to influence desmosomal cadherin gene expression in mouse keratinocytes (Smith, Zhu et al. 2004). The C/EBPs belong to the basic leucine-zipper class of transcription factor (Vinson, Sigler et al. 1989). To date, six members (C/EBP α - ζ) of this family have been described, which share both structural and functional similarities (Lekstrom-Himes and Xanthopoulos 1998). Expression of the C/EBP isoforms has been shown to change throughout the development of a number of different tissues, as well as in response to injury (Ramji and Foka 2002). The C/EBPs appear to play a role in the regulation of gene expression in colonic development. C/EBP α is involved in growth arrest and terminal differentiation in the embryonic mouse intestine (Chandrasekaran and Gordon 1993) and C/EBP α , C/EBP β and C/EBP δ all demonstrate altered expression patterns in the post-natal development of murine colonic cells (Blais, Boudreau et al. 1995).

The aim of the experiments described in this chapter was to determine whether C/EBPs are important in the regulation of *DSC2* and *DSG2* gene expression in the colon. Firstly, the effect of C/EBPs on desmosomal cadherin promoter activity in the colonic cancer cell line, Caco2 was explored. To achieve this, fragments of the *DSC2* and *DSG2* promoters were cloned into a vector containing a luciferase reporter gene. DNA encoding various C/EBP isoforms was then co-transfected into Caco2 cells with the reporter constructs, and their effect on luciferase activity measured. Secondly, experiments were carried out to investigate the possibility that up-regulation of C/EBPs in colorectal cancer is responsible for the aberrant expression of *DSC1* and *DSC3* that occurs in colorectal cancer specimens (Chapter 3). Finally, investigations were performed to determine the role of other transcription factors with putative binding sites in the *DSC2* and *DSG2* promoters in the regulation of expression of these genes in Caco2 cells.

5.2 Identification of consensus binding sites within the *DSC2* and *DSG2* promoters

The promoter sequences of the desmosomal cadherin genes *DSC2* (accession number Y08431) and *DSG2* (accession number AJ278448) was scrutinised using MatInspector v2.2 software (Quandt, Frech et al. 1995), in order to identify putative transcription factor binding sites. The sequences of over 100 binding sites were identified in this manner, including those for C/EBPs, AP1, AP2, AP4, TCF/LEF1 and p53 (Tables 5.1 and 5.2).

Table 5.1 Positions of putative transcription factor binding sites relative to the ATG initiation codon in the *DSC2* promoter

Transcription Factor	Position
C/EBP	-285, -665, -735, -825, -928
AP1	-452, -541, -585, -611, -695, -751, -863, -924
AP2	-60, -505, -538
AP4	-99, -359, -443, -508, -594
TCF-LEF1	-165, - 695
p53	-20, -433, -500, -757

Table 5.2 Positions of putative transcription factor binding sites relative to the ATG initiation codon in the *DSG2* promoter

Transcription Factor	Position
C/EBP	-645, -666, -808, -935, -957
AP2	-31, -101, -181, -549,
TCF-LEF1	-844, -895
p53	-686, -691, -702

NB. No binding sites were identified for AP1 or AP4 within the 1000 bases proximal to the ATG codon.

Fig 5.1 Location of the primers used to amplify fragments of the *DSC2* promoter

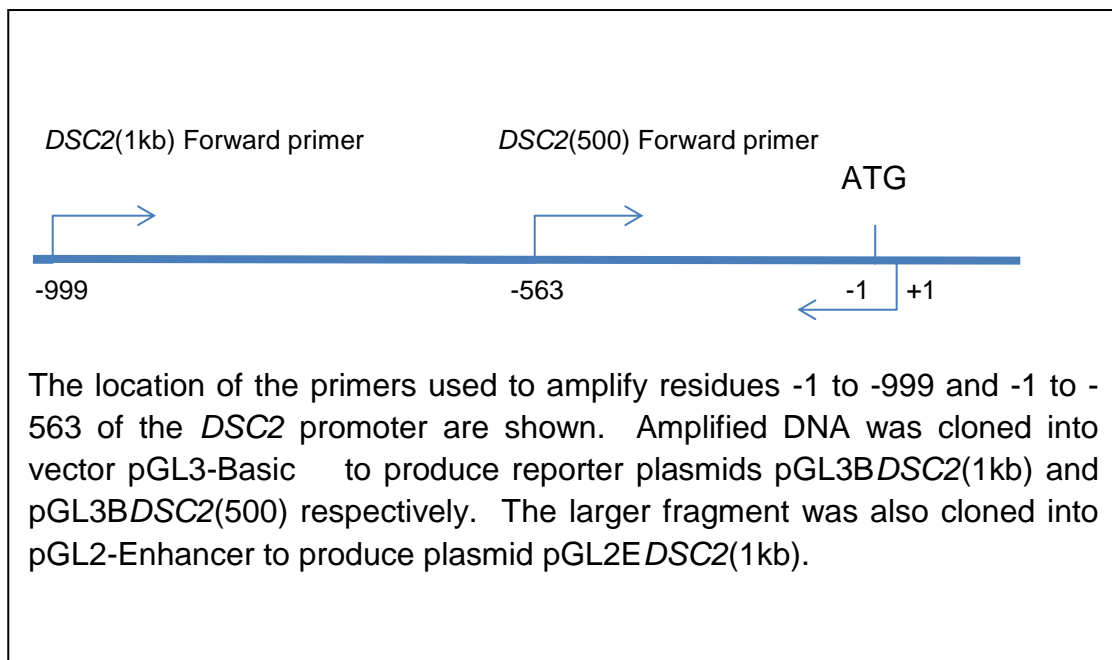
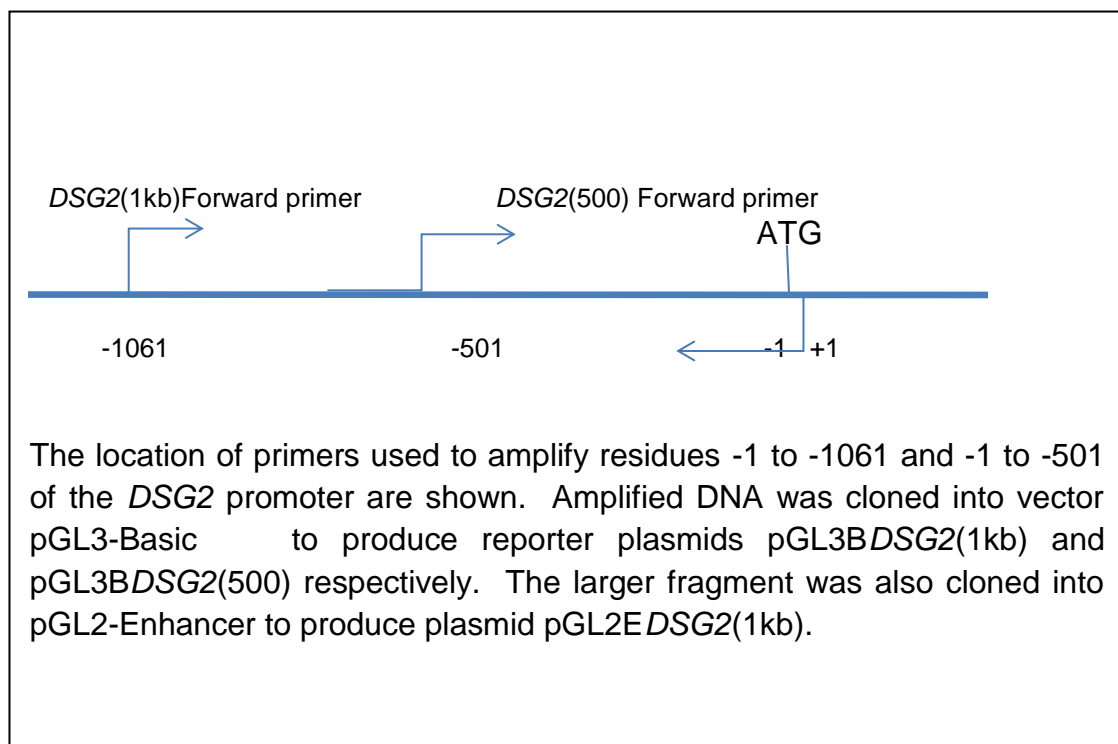


Fig 5.2 Locations of the primers within the *DSG2* promoter sequence

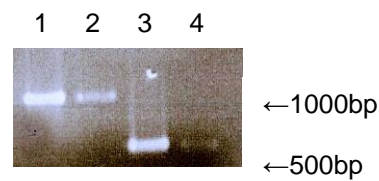


5.3 Construction of reporter plasmids

DNA was prepared from the yeast artificial chromosome (YAC) clones 14E-B5 (*DSC2*) and 9G-C3 (*DSG2*). Fragments upstream of the *DSC2* and *DSG2* genes were then amplified by PCR using YAC DNA as starting material and the oligonucleotides described in Table 2.5 (and shown in Figures 5.1 and 5.2). Figure 5.3 shows the results of the PCR amplification of the *DSC2* promoter fragments.

DSC2 and *DSG2* PCR products were cut with *KpnI*-*XhoI* and ligated into *KpnI*-*XhoI* cut pGL3Basic reporter plasmid, to yield pGL3BD*DSC2*(1kb), pGL3BD*DSC2*(500), pGL3BD*DSG2*(1kb) and pGL3*DSG2*(500). Similarly, *KpnI*-*XhoI* fragments were inserted into *KpnI*-*XhoI* cut pGL2Enhancer to yield pGL2ED*DSC2*(1kb) and pGL2ED*DSG2*(1kb). All inserts were sequenced to ensure that no mutations had been inadvertently introduced during PCR amplification. The sequenced plasmid DNA was then used in transient transfection experiments in cultured Caco2 cells.

Fig 5.3 Amplification of the *DSC2* promoter by PCR



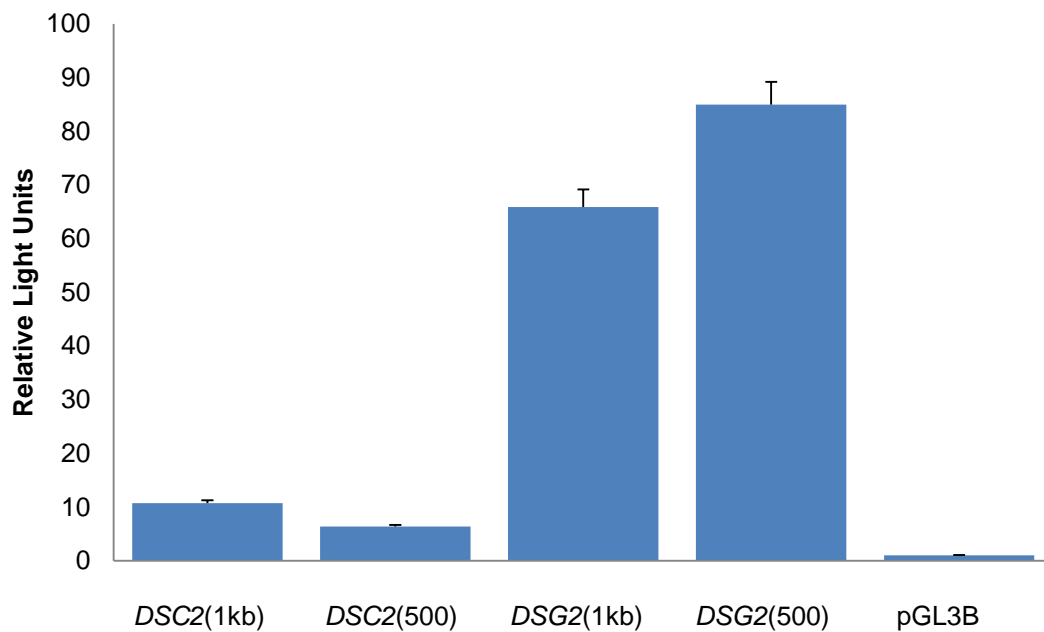
Agarose gel showing the PCR products obtained from the *DSC2* YAC clone 14E-B5 to yield two fragments of the *DSC2* promoter, 998 bases (lanes 1 and 2) and 562 bases (lanes 3 and 4) upstream of the ATG initiation codon. The two PCR reactions were carried out with two different DNA concentrations: 10ng per reaction (lanes 1 and 3) and 1ng per reaction (lanes 2 and 4). 12.5 μ l of each PCR reaction was loaded onto the gel.

5.4 Transfections of Caco2 cells with reporter constructs

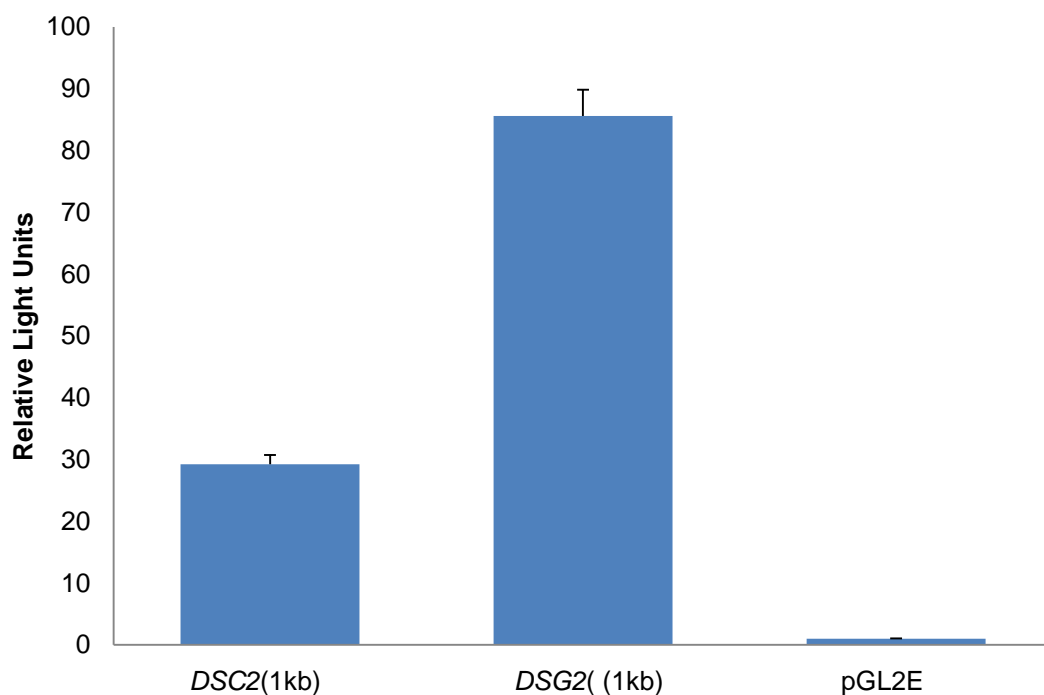
Initial experiments were used to establish baseline activity of the promoter constructs in the colonic cancer cell line, Caco2. The pGL3-Basic plasmids contain no eukaryotic promoter or enhancer sequences. Thus insertion of the promoter element under investigation upstream of the luciferase gene allows its activity to be monitored by measuring luciferase expression. The pGL2-Enhancer vector contains an SV40 enhancer element downstream of the luciferase gene, which results in increased luciferase transcription. The luciferase activity was greater with the enhancer constructs, however adequate luciferase activity was recorded with all the pGL3-Basic promoter constructs (Fig 5.4) and therefore the pGL3-Basic reporter plasmids were used in preference to the pGL2-Enhancer reporter constructs for the subsequent experiments. Both *DSG2* constructs showed greater baseline activity than the *DSC2* plasmids. The longer *DSC2*(1kb) fragment showed greater activity than the *DSC2*(500) fragment, however the opposite was observed with the *DSG2* constructs. This may be due to the presence of negative regulatory elements situated in the distal portion of the *DSG2* (1kb) promoter fragment.

Fig 5.4 Baseline luciferase activity in *DSC2* and *DSG2* reporter plasmids

a. pGL3-Basic reporter plasmids



b. pGL2-Enhancer reporter plasmids



Luciferase reporter plasmids were constructed using fragments of the *DSC2* and *DSG2* promoters. Caco2 cells were transiently transfected with reporter plasmids and firefly-luciferase activity measured after 48h. Transfection efficiency was monitored with plasmid pRL-TK (Fig 2.3). Each bar represents the means \pm SD for two experiments carried out in triplicate.

5.5 Effect of C/EBPs on *DSC2* promoter activity

To examine the effect of C/EBPs on desmosomal cadherin promoter activity reporter constructs were co-transfected into Caco2 cells with pcDNA-based expression plasmids encoding a variety of C/EBP transcription factors. In initial experiments the longer *DSC2* reporter construct, containing bases -1 to -999 of the *DSC2* promoter, was used. C/EBPs α and ζ (CHOP) had no effect on luciferase activity when compared to the empty expression vector pcDNA3 (Fig 5.5a). However, both C/EBP β and C/EBP δ had a significant activating effect, with C/EBP β causing a 4.2-fold increase in promoter activity and C/EBP δ causing a 1.8-fold increase in activity. With the shorter *DSC2* reporter construct, containing bases -1 to -563 of the *DSC2* promoter, C/EBP β retained its transactivating ability, albeit with 30% reduction (Fig 5.5b). This suggests that an important binding site for C/EBP β lies in the first 563 bases upstream of the ATG codon. The ability of C/EBP δ to activate the *DSC2* promoter was abrogated with the shortened reporter plasmid, suggesting that transactivation by C/EBP δ is due to interactions taking place at a binding site in the distal part of the *DSC2* (1kb) promoter construct.

5.6 Effect of C/EBPs on *DSG2* promoter activity

C/EBPs α , β , and δ all significantly activated the *DSG2*(1kb) promoter, with C/EBP α responsible for a 2.2-fold increase in activity, C/EBP β responsible for a 3.8-fold increase in activity and C/EBP δ responsible for a 2.7-fold increase in activity (Fig 5.6a). Interestingly, C/EBP ζ (CHOP) significantly repressed the *DSG2* promoter to only 60% of the baseline activity. C/EBP ζ shares the

leucine zipper with other C/EBP members and thus can form heterodimers with them. But although C/EBP ζ is able to interact with other C/EBP family members, it lacks the C/EBP DNA-binding recognition sequence and thus acts as a functional repressor of C/EBP activity (Ron and Habener 1992). Thus it may be that endogenous Caco2 C/EBPs contribute to promoter activity that is seen in the absence of exogenous transcription factors (Fig 5.4) and that these are inhibited by exogenous C/EBP ζ . When the reduced length promoter construct was used in transfection experiments, the effects seen with all the C/EBPs tested were decreased (Fig 5.6b). Only C/EBP β showed statistically significant activity above baseline. This activity was reduced by approximately 30% when compared to the *DSG2*(1kb) promoter construct. MatInspector only identified C/EBP binding sites within the distal part of the *DSG2* promoter sequence so it may be that C/EBP β transactivates the *DSG2*(500) reporter plasmid via a site that is not recognised by the programme.

Fig 5.5 Effect of C/EBPs on DSC2 promoter activity

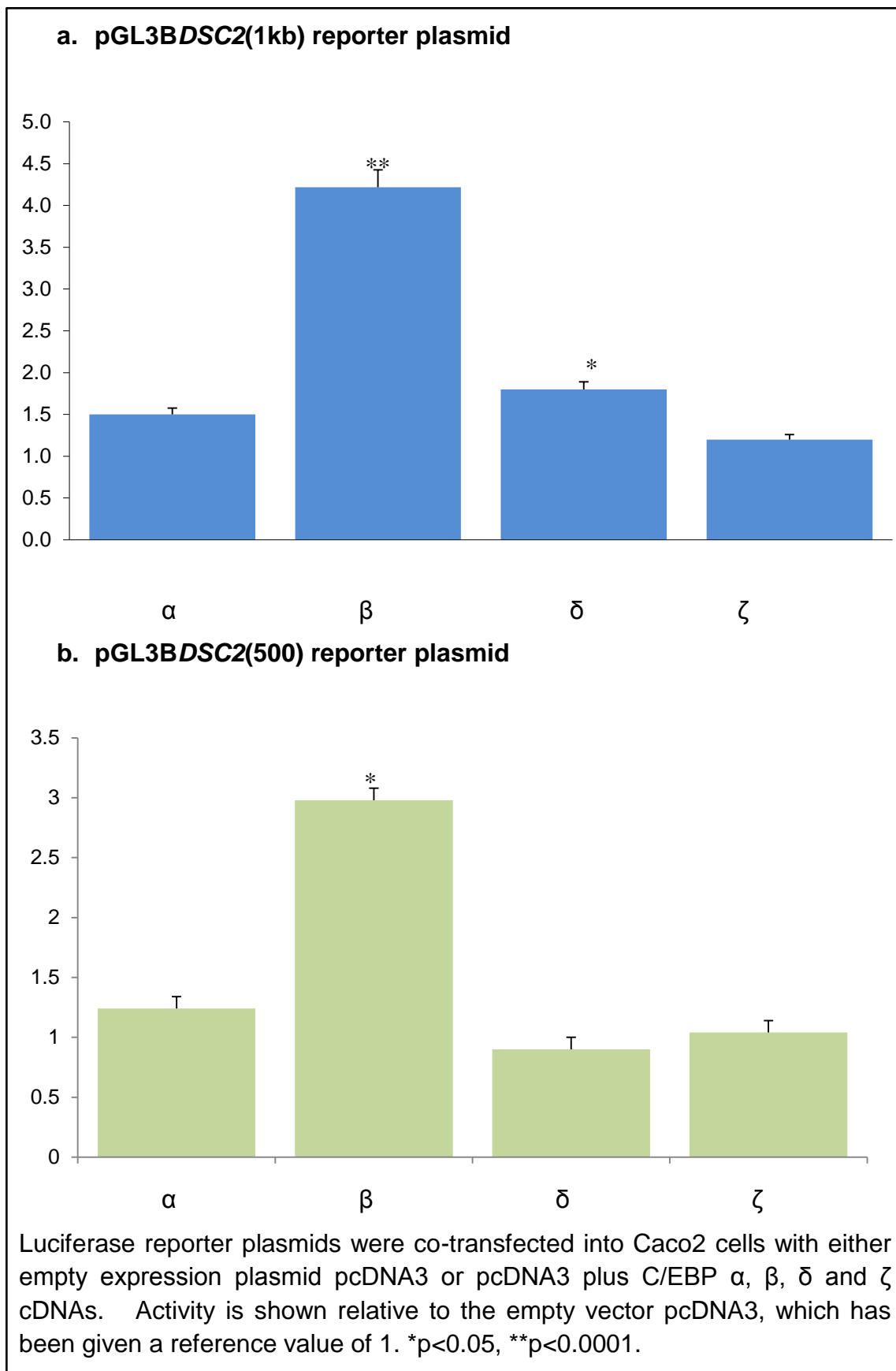
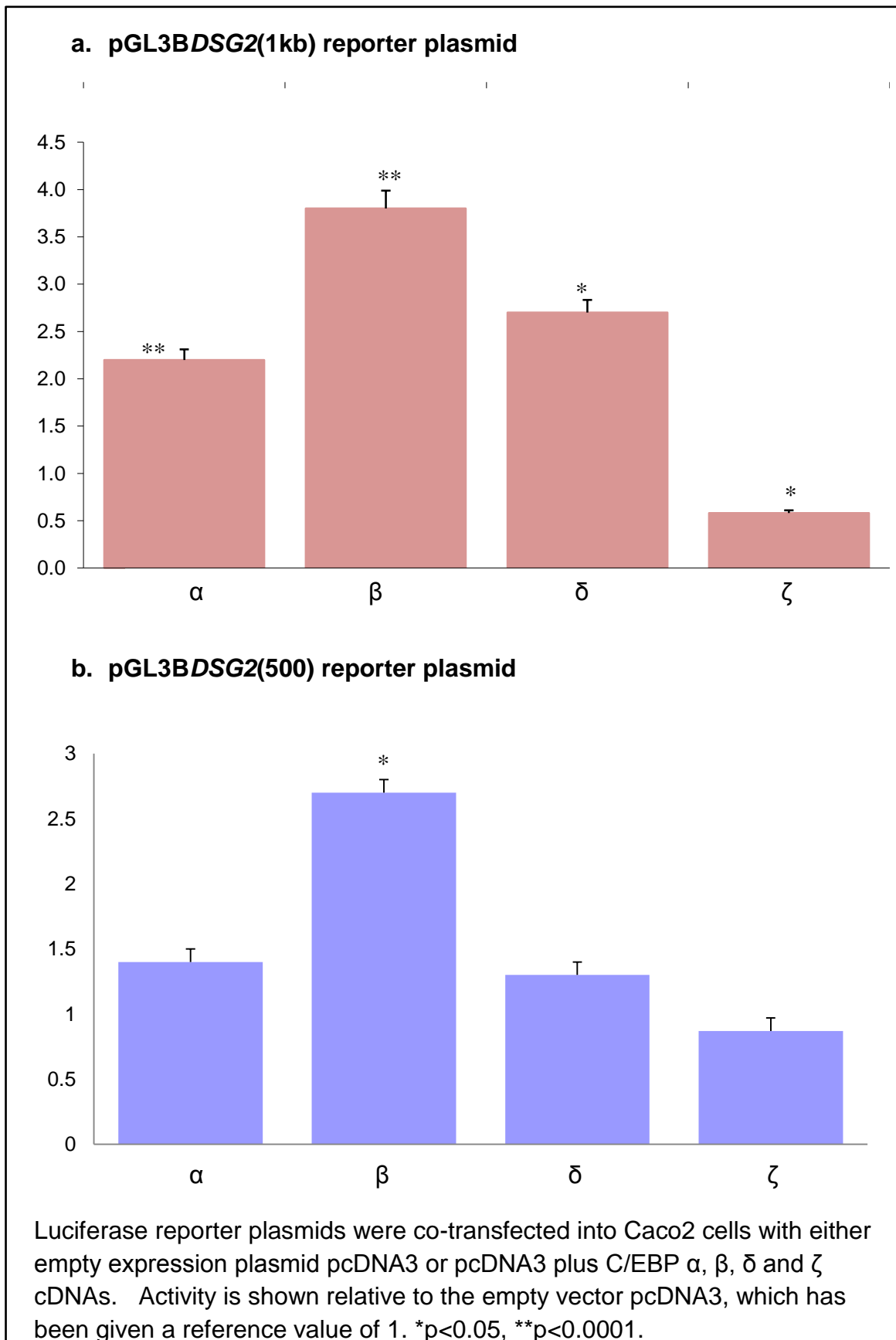


Fig 5.6 Effect of C/EBPs on *DSG2* promoter activity



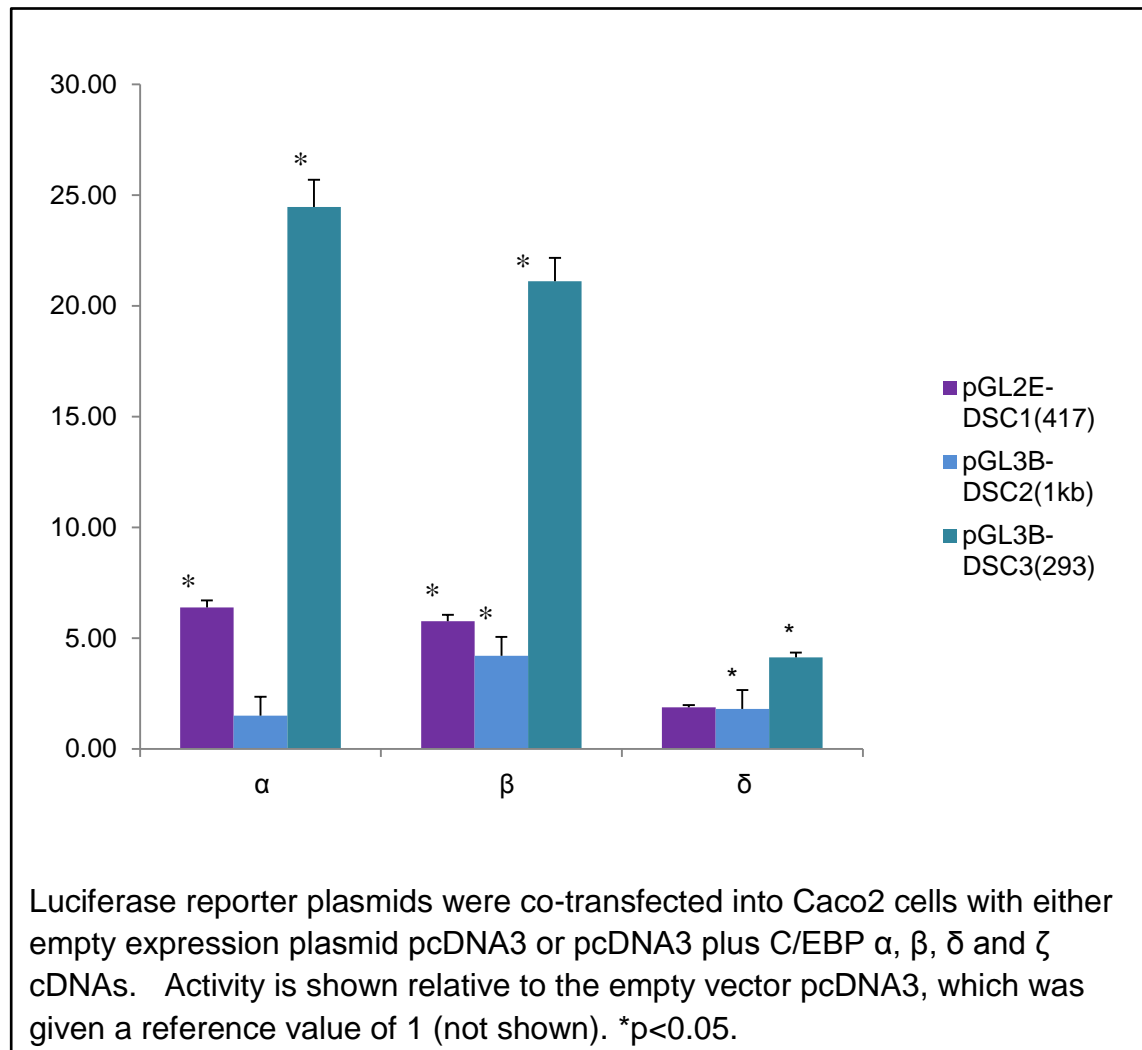
5.7 Effect of C/EBPs on *Dsc1* and *Dsc3* promoter activity in Caco2 cells

Having previously demonstrated that the desmosomal cadherins not normally expressed in colonic cells, are aberrantly expressed in colorectal cancer (Chapter 3), we carried out preliminary experiments to identify transcription factors that may activate *DSC1* and *DSC3* gene expression in colorectal cancer cells. Human *DSC1* and *DSC3* promoter constructs were not available so as a first step we used murine promoter constructs that had been used in a previous study on the regulation of *Dsc1* and *Dsc3* activity in the epidermis (Smith, Zhu et al. 2004). In the case of *Dsc1* the construct consisted bases -1 to -417bp of the mouse *Dsc1* gene cloned into the plasmid pGL3-Enhancer, upstream of the luciferase reporter gene. A similar construct containing bases -1 to -293 from mouse *Dsc3* in pGL3-Basic was used.

C/EBP α and C/EBP β both significantly stimulated transcription from the *Dsc1* and *Dsc3* promoters in human Caco2 cells. C/EBP δ also activated the *Dsc3* promoter but not the *Dsc1* promoter. C/EBP α had the greatest effect out of the three C/EBPs tested, on both the *Dsc1* and *Dsc3* promoters. Although C/EBPs influence *DSC2* promoter activity, it appears that they have a much greater effect on desmocollins not typically expressed in normal colonic epithelium. The *Dsc3* promoter appeared to be the most influenced by the C/EBPs, showing a 20-fold increase and 24-fold increase above base-level in response to C/EBP α and C/EBP β respectively. Although preliminary, these results do show a potential mechanism for the activation of desmosomal cadherins in colorectal cancer as up-regulation of either C/EBP α or β in

colorectal cancer cells could potentially lead to a dramatic increase in expression of DSC3 in particular.

Fig 5.7 Effect on C/EBPs on *Dsc1*, *DSC2* and *Dsc3* promoter activity

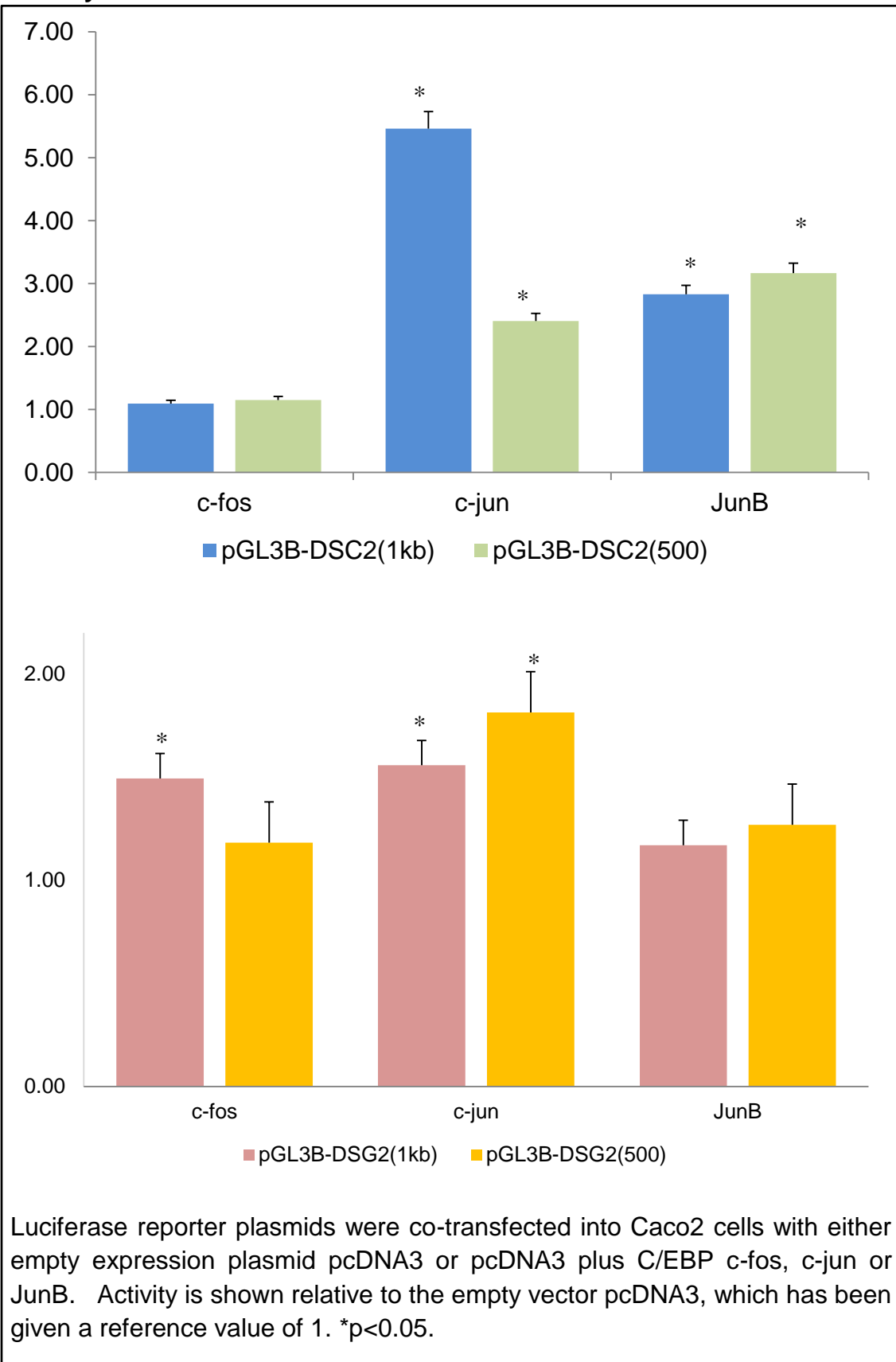


5.8 Effect of activator protein-1 (AP1) transcription factors on *DSC2* and *DSG2* promoter activity

Putative binding sites for a variety of transcription factors were detected in the *DSC2* and *DSG2* promoters by the MatInspector programme (Tables 5.1 and 5.2). Hence experiments were performed to compare the transactivating ability of these transcription factors with that of the C/EBPs. A number of AP1 binding sites were identified by the MatInspector programme in the *DSC2* promoter (Table 5.1), although not the *DSG2* promoter (Table 5.2). The AP1 transcription factor is made up of basic leucine zipper proteins from the Fos families (c-fos, FosB, Fra-1 and Fra-2) and the Jun families (c-Jun, JunB and JunD) which join together as either homodimers or heterodimers, to yield a variety of responses. It has been implicated in cell cycle control, cell proliferation and apoptosis, as well as malignant transformation and resistance to chemotherapeutic agents in colorectal cancer (Ashida, Tominaga et al. 2005).

When co-transfected into Caco2 cells with *DSC2* reporter constructs both c-fos and JunB had a significant activating effect (Fig 5.8). c-Jun caused a 5.5-fold activation of the *DSC2*(1kb) promoter and a 2.5-fold activation of the *DSC2*(500) promoter. Truncation of the *DSC2* promoter had little effect on the transactivating ability of JunB which caused a 3-fold activation of the longer *DSC2* promoter construct and a 3.2-fold activation of the shorter *DSC2* promoter construct. The effect of AP1 factors on the *DSG2* promoter was less dramatic with c-fos and c-jun, but not JunB, causing moderate (<2-fold) increases in transcriptional activity.

Fig 5.8 Effect of AP1 transcription factors on *DSC2* and *DSG2* promoter activity



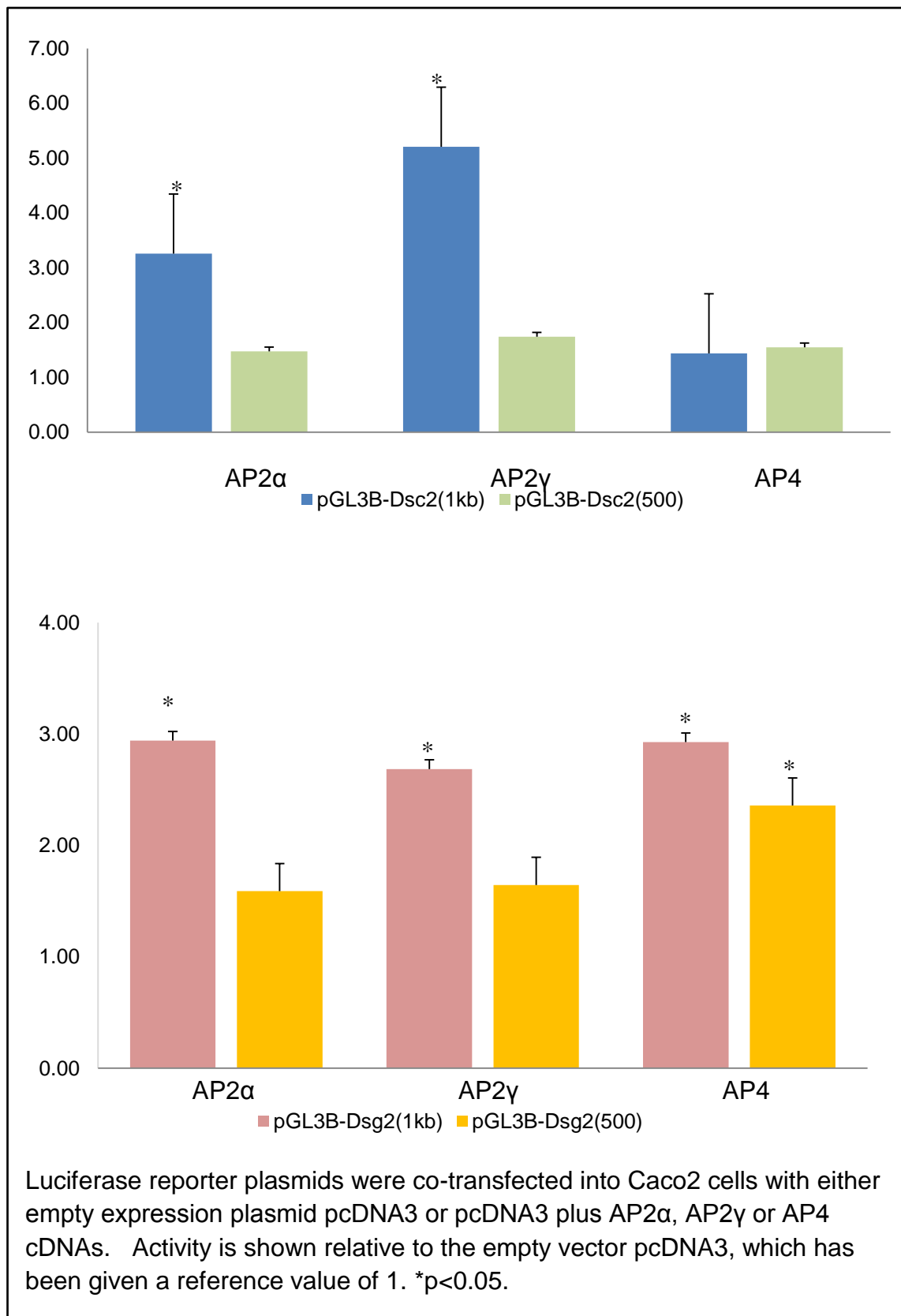
5.9 Effect of activator protein-2 (AP2) and activator protein-4 (AP4) transcription factors on *DSC2* and *DSG2* promoter activity

Putative binding sites for transcription factor AP2 were located in both the *DSC2* and *DSG2* promoters by the MatInspector programme (Tables 5.1 and 5.2). A number of potential binding sites for the AP4 transcription factor were also located in the *DSC2*, but not the *DSG2* promoter. AP2 is involved in the regulation of cell proliferation, differentiation and apoptosis, and has been implicated in the development of cancer. AP2 α is thought to act as a tumour suppressor in many cancers and it is thought that AP2 γ may act as a prognostic marker in testicular cancer (Pellikainen and Kosma, 2007). AP4 is a transcription factor normally only expressed in the non-differentiated, proliferative progenitor compartment of colonic crypts. Expression of AP4 has been shown to correlate with colorectal cancer progression and the presence of lymph node metastasis (Cao, Tang et al. 2009).

Both AP2 α and AP2 γ were shown to stimulate the *DSC2* promoter, causing a 3.3-fold and a 5.2-fold increase in luciferase activity (Fig 5.9). The transactivating effect was lost when the distal 500 bases of the promoter were deleted. AP4 had no effect on the *DSC2* promoter, despite a number of putative AP4 sites being identified by the MatInspector programme. A similar pattern was seen with the *DSG2* constructs, with AP2 α and AP2 γ causing 2.9- and 2.7-fold increases in luciferase activity respectively with the longer promoter construct. In the case of the *DSG2* promoter AP4 did stimulate a 2.4-fold increase in activity with the longer construct, presumably through

binding to a site not identified by MatInspector. This effect was maintained in the shorter construct.

Fig 5.9 Effect of AP2 and AP4 on *DSC2* and *DSG2* promoter activity



5.10 Effect of β -catenin, plakoglobin and p53 on *DSC2* and *DSG2* promoter activity

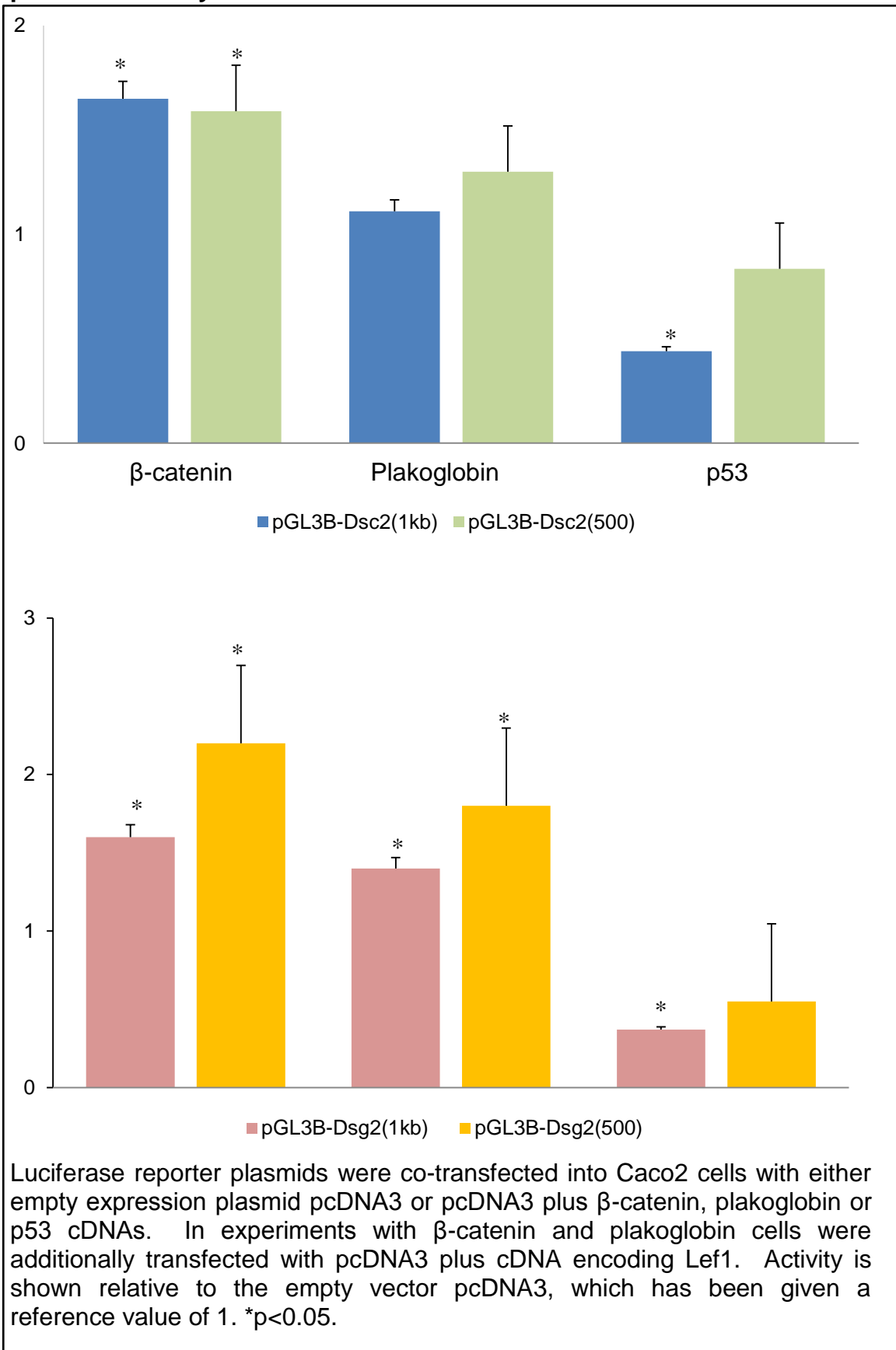
The T-cell factor/Lymphoid enhancing factor-1 (Tcf/Lef) family is a group of transcription factors that form complexes with β -catenin, an Armadillo family member that plays a key role in the Wnt signalling pathway (Behrens, von Kries et al. 1996; van de Wetering, Cavallo et al. 1997). Tcf/Lef- β -catenin complexes activate transcription and failure to degrade β -catenin is associated with the development of most colorectal tumours (Morin, Sparks et al. 1997). Plakoglobin is related to β -catenin and interacts with many of the same proteins. However, its role in Wnt/ β -catenin signalling is less well defined; the possibility exists that plakoglobin may act as both a positive and negative regulator of Wnt signalling (Chidgey and Dawson, 2007). The tumour suppressor gene, p53 is located on chromosome 17p, a location often lost or altered in many malignancies, including colorectal cancer (Vogelstein, Fearon et al. 1988). It has numerous roles as a transcriptional regulator and has been shown to regulate desmocollin gene expression in both breast and colorectal cancer cells (Oshiro, Watts et al. 2003; Cui, Chen et al. 2011).

A small but significant 1.7-fold activation of the *DSC2* promoter was achieved by transient transfection of Caco2 cells with β -catenin (Fig 5.10). As β -catenin acts in concert with Tcf/Lef family members, the cells were additionally transfected with Lef1, which had no significant effect on its own (data not shown). The transactivating effect of β -catenin was maintained when the experiment was repeated with the shorter *DSC2* promoter plasmid.

Co-transfection of plakoglobin and Lef1 had no significant effect on luciferase activity. Significant repression of the *DSC2* promoter was obtained with p53 although this effect was lost with *DSC2*(500) construct (Fig 5.10). This was somewhat surprising given that a number of putative p53 binding sites were located by the MatInspector programme in the proximal *DSC2* promoter (Table 5.1).

Both β -catenin (1.6-fold) and plakoglobin (1.4-fold) had a modest transactivating effect on the longer *DSG2* promoter construct (Fig.5.10). The transactivating effect was maintained and somewhat enhanced when the shorter reporter construct was used. As with *DSC2*, *DSG2* promoter activity appears to be repressed by p53. This effect was attenuated with the shorter promoter construct.

Fig 5.10 Effect of β -catenin, plakoglobin and p53 on *DSC2* and *DSG2* promoter activity



5.11 Discussion

The experiments of chapter 2 demonstrated that DSC2 and DSG2 expression is lost in colorectal cancer, and that other desmosomal cadherins (DSC1, DSC3, DSG1, and DSG3) are aberrantly expressed. One potential explanation for these observations is that changes in desmosomal cadherin gene expression occur as a result of abnormal expression of transcription factors in colorectal cancer. The experiments described in this chapter were designed primarily to identify transcription factors that regulate desmosomal cadherin gene expression in colonic epithelial cells. The C/EBP family of transcription factors provide the starting place for this investigation as they have previously been shown to play a role in the regulation of murine *Dsc1* and *Dsc3* gene expression in keratinocytes (Smith, Zhu et al. 2004).

The C/EBPs belong to the basic leucine zipper class of transcription factors, and consist of an activation domain, a DNA-binding basic region and a leucine-rich dimerisation domain (Agre, Johnson et al. 1989). Dimerisation takes place via the leucine zipper, to form partnerships between the C/EBP family members, as well as with other transcription factor families, such as NFκB and the AP1 factors (Vinson, Hai et al. 1993). Dimerisation is essential to the DNA binding process (Landschulz, Johnson et al. 1989), but the ability of hetero- and homo-dimers to transactivate is variable (Poli, Mancini et al. 1990; Kinoshita, Akira et al. 1992).

The current study demonstrated for the first time that the *DSC2* and *DSG2* promoters, in common with the *Dsc1* and *Dsc3* promoters (Smith, Zhu et al. 2004), are transactivated by C/EBPs. In mouse keratinocytes *Dsc1* is most strongly activated by C/EBP α , whereas *Dsc3* is most strongly activated by C/EBP β (Smith, Zhu et al. 2004). In human Caco2 cells the *DSC2* and *DSG2* promoters are most strongly activated by C/EBP β . Both C/EBP α and C/EBP β have very strong transactivating effects on the murine *Dsc3* promoter in Caco2 cells, although whether the same is true of human *DSC3* promoter remains to be seen. High levels of C/EBP β have been observed in colorectal cancer (Rask, Thörn et al. 2000) and it is conceivable that it is responsible for the de novo expression of *DSC3* observed in colorectal cancer specimens (chapter 3) although much more work will be required to definitively prove that there is a causal link between the two.

Other than C/EBPs, a number of other transcription factors were identified that could be important in the regulation of desmosomal cadherin gene expression in colorectal cells. Of these the effect of c-jun and AP2 γ on the *DSC2* promoter was the most dramatic (Fig 5.8 and Fig 5.9). Neither of these transcription factors has been previously implicated in the regulation of desmosomal cadherin gene expression and further investigation would be required (see below) to confirm their role. Interestingly, of the transcription factors tested only p53 repressed transcription from the *DSC2* and *DSG2* promoters (Fig 5.10). Loss of p53 expression, a frequent event in colorectal cancers, might therefore be expected to result in an increase in *DSC2* and

DSG2 expression, the opposite of our findings in Chapter 2. Nevertheless it would be interesting to determine the effect of p53 on other desmosomal cadherin promoters, particularly *DSC3* which has been previously identified as a p53-target gene (Oshiro, Watts et al., 2003; Cui, Chen et al., 2011).

Clearly much of the work described in this chapter is of a preliminary nature and much more experimental data are required. Confirmation of a role for C/EBPs, and other transcription factors such as c-jun and AP2 γ , in the regulation of desmosomal cadherin gene expression could be obtained using techniques such as electrophoretic mobility shift assays (EMSA) and chromatin immunoprecipitation (ChIP) assays. EMSA assays can be used to confirm whether transcription factors are able to bind to particular sequences of DNA (such as those identified in the *DSC2* and *DSG2* promoters by the MatInspector programme) *in vitro*. ChIP assays can be used to determine whether transcription factors such as the C/EBPs are able to interact with desmosomal cadherin promoters *in vivo*. Further reporter assays could be carried out with mutated promoter sequences to confirm the importance of putative binding sites in promoter regions.

It would be interesting to examine the expression patterns of C/EBPs, particularly C/EBP α and C/EBP β in colorectal cancer specimens, and to examine the role of C/EBPs on human, as opposed to murine, promoters in colorectal cells. It may be that other mechanisms are responsible for

silencing *DSC2* and *DSG2* expression in colonic cancer cells. The mechanism by which many genes are silenced in colorectal cancer is by hypermethylation of CpG islands which are abundant in the promoter regions of some genes. The *DSC2* promoter contains a 500bp CpG island (Marsden, Collins et al., 1997) so it would be useful to examine the methylation status of *DSC2*, and other desmosomal genes, in colorectal cancer.

CHAPTER 6

DISCUSSION

6.1 Introduction

This investigation has shown for the first time altered desmocollin expression in colorectal cancer. At the protein level two distinct changes were observed, namely loss of desmocollin 2, together with gain of expression of desmocollin 1 and desmocollin 3, desmosomal cadherins not normally expressed in colonic mucosa. At the transcriptional level no change was observed in *DSC2* mRNA. However, the gain of expression of *DSC1* and *DSC3* was accompanied by increased message levels. Desmoglein protein levels were not examined but increased levels of mRNA encoding desmogleins not normally expressed in colonic mucosa (i.e. *DSG1*, *DSG3* and *DSG4*) were observed, although there was no change in levels of mRNA encoding *DSG2*, the desmoglein normally expressed in colonic mucosa. An attempt was made to investigate the effects of loss of desmosomal cadherin gene expression on the behaviour of cultured cells, and potential mechanisms that could account for the changes in gene expression were explored. The aim of this final chapter is to discuss these findings in the context of current evidence and knowledge of the role of desmosomal cadherins in colorectal cancer, as well as to consider future avenues of investigation.

6.2 Changes in desmocollin and desmoglein gene expression in colorectal cancer

6.2.1 Changes in protein expression

Changes in desmocollin expression patterns in colorectal cancer may result in significant alterations in desmosome function. In the healthy colonic mucosa, DSC2 and DSG2 (Nuber, Schafer et al. 1995) are the only desmosomal cadherins that are expressed and therefore loss of these adhesion molecules could manifest itself as altered behaviour of colonic cells. Loss of DSG2-mediated adhesion has been shown to interfere with intestinal epithelial barrier function, altering gut permeability and potentially allowing pathogens to translocate systemically (Schlegel, Meir et al. 2010). Other cancers have demonstrated loss of desmosomal components, which may correlate with disease progression. For example, reduced DSC2 expression correlates with poor prognosis in pancreatic adenocarcinoma (Hamidov, Altendorf-Hofmann et al. 2012).

Three changes in desmosomal cadherin protein expression were observed in this series of experiments: reduced DSC2 expression; redistribution of DSC2 away from the cell surface to the cytoplasm; and expression of DSC1 and DSC3, which are not normally expressed in the colonic epithelium. Although reduced DSC2 protein expression was observed in colorectal cancer samples this was not due to decreased transcription, as the *DSC2* mRNA was unchanged in the majority of samples examined. It may be that DSC2 protein is lost because of decreased rates of protein synthesis or increased protein

degradation. Loss of protein stability as a result of mutations is one possibility although it should be noted that no mutations in desmocollins have been documented in colorectal or any other type of cancer. Several studies have shown desmogleins and desmocollins to be targeted by caspases and matrix metalloproteinases during desmosome breakdown (Weiske, Schoeneberg et al. 2001; Cirillo, Lanza et al. 2008; Kami, Chidgey et al. 2009), and it may be that dysregulation of this system in colorectal cancer results in loss of DSC2 protein.

Drawing a comparison with the classical cadherins, loss of E-cadherin during TGF/Mitogen activated protein kinase (MAPK)-induced epithelial-mesenchymal transition (EMT) not only takes place through reduced transcription but also by post-translational mechanisms including internalisation and endocytosis (Janda, Nevolo et al. 2006). Protein kinase C has also been shown to lead to the internalisation of classical cadherins (Le, Joseph et al. 2002) and given that PKC is thought to be involved in desmosome remodelling (Wallis, Lloyd et al. 2000), it is possible that the relocalisation of desmosomal cadherins seen in this study is due to similar processes. In addition, tyrosine phosphorylation of DSG2, as seen in cells over-expressing the epidermal growth factor receptor (EGFR), has been shown to result in reduced levels of both DSG2 and DSC2 at the cell membrane, whilst increasing the internalised pool of desmosomal cadherins (Lorch, Klessner et al. 2004). Furthermore, Windoffer et al observed that desmosome stability was diminished when extracellular calcium was reduced

and that DSC2 was translocated from the cell membrane to the cytoplasm, where it remained (Windoffer, Borchert-Stuhltrager et al. 2002). Even when the calcium levels were restored, it appears that the desmosomal cadherins previously engaged in desmosome formation are not re-utilised, suggesting that a new source of desmosomal cadherins is made available to support desmosome formation. Studies investigating the assembly and stability of desmosomal components have shown desmosomal cadherins to be much more stable when incorporated into desmosomes and when free, are rapidly degraded (Pasdar and Nelson 1989).

De novo DSC1 and DSC3 staining was observed both at the cell membrane and in the cell cytoplasm. It may be that these desmocollins simply replace DSC2 in terms of its adhesive function. However, an alternative explanation could be that rather than acting as a compensatory mechanism, the presence of DSC1 and DSC3 at the cell membrane may lead to the three desmocollins 'competing' with each other to be incorporated in the junctional structure, which could result in DSC2 being displaced from the desmosome, relocalised to the cytoplasm and degraded by endocytosis. It is not known whether the three desmocollins are equally adhesive, or whether there are subtle differences in adhesive function that could lead to changes in cell behaviour. Incorporation of different isoforms into desmosomes appears to be isoform-dependent and can lead not only to desmosome dissociation but also to alterations in plakoglobin expression (Ishii, Norvell et al. 2001).

The immunohistochemistry study also showed that all samples with decreased DSC2 expression exhibited diminished E-cadherin expression (Khan, Hardy et al. 2006). E-cadherin is a classical transmembrane cadherin found in adherens junctions. It has a well-established tumour suppressor role, with loss of E-cadherin function having been demonstrated in gastric cancer, invasive lobular breast cancer, prostate and colorectal cancer (Hirohashi 1998; Kuphal and Behrens 2006). Communication between adherens junctions and desmosomes takes place during development, with E-cadherin directing desmosome formation (Gumbiner, Stevenson et al. 1988). Plakoglobin is thought to mediate this cross-talk as it has been shown to incorporate into both desmosomes and adherens junctions (Lewis, Wahl et al. 1997). E-cadherin expression is thought to contribute to the stability of desmosomal cadherins, which are rapidly degraded when cell contacts are diminished (Penn, Burdett et al. 1987) so it may be that loss of E-cadherin in colorectal cancer may cause disruption of desmosomes. However the relationship appears to be co-operative, as desmosomal components also contribute to the formation of mature adherens junctions (Vasioukhin, Bowers et al. 2001). Coordinated expression of both adherens junctions and desmosomes is able to significantly strengthen intercellular adhesion (Getsios, Huen et al. 2004) and it may be that both are required to prevent changes in cell behaviour.

Desmosomal adhesion differs from that exhibited by adherens junctions. In tissues or cells that have been cultured for long periods desmosomes are able

to achieve a hyper-adhesive state in which they become stable even in the absence of extracellular calcium (Borysenko and Revel 1973; Kimura, Merritt et al. 2007). Adherens junctions are unable to achieve a hyperadhesive state and remain sensitive to extracellular calcium levels, even after prolonged periods of cell-cell contact. Calcium-induced cell contact has been shown to increase the metabolic stability of some of the individual desmosomal components, including desmogleins (Pasdar and Nelson 1989). In turn, this may influence the stability of other components, as the degradation of plakoglobin is greatly reduced by co-expression of desmogleins and desmocollins (Kowalczyk, Palka et al. 1994). The switch between the hyperadhesive calcium-independent state and a calcium-dependent state has been observed as a response to wounding (Garrod, Berika et al. 2005) and is thought to be mediated by protein kinase C (Wallis, Lloyd et al. 2000). By allowing desmosomes to disassemble and migrate into the wound, PKC facilitates epithelial remodelling and repair. Although PKC has been shown to reduce colonic cell proliferation via suppression of β -catenin signalling (Gwak, Jung et al. 2009), it also is able to promote desmosome assembly in an α -catenin deficient colon cancer cell line (Hengel, Gohon et al. 1997).

The desmocollins are thought to have an early role in desmosome assembly, reaching the cell surface before desmogleins (Burdett and Sullivan 2002). Furthermore, in keeping with the observations that desmosomal components influence adherens junction assembly, loss of DSC3 function appears to

interfere with both desmosome and adherens junction formation, whereas loss of DSG3 function only affects desmosomes (Hanakawa, Amagai et al. 2000). Therefore DSC2 loss, as demonstrated in this investigation may affect intercellular adhesion mediated by both desmosomes and adherens junctions.

6.2.2 Changes in mRNA expression

Despite the reduction of DSC2 protein, no loss of *DSC2* mRNA expression was observed. However, de novo expression of *DSC1* and *DSC3* mRNA was identified in the tumour samples studied. As discussed previously, all desmosomal cadherins lie close together on chromosome 18q and share significant sequence homology (Hunt, Sahota et al. 1999). Expression of the different isoforms within a tissue appears to take place in a coordinated fashion (King, Angst et al. 1997; Smith, Zhu et al. 2004) suggesting these events are linked. Loss of DSC2 protein may trigger a compensatory activation of *DSC1* and *DSC3* gene transcription (Hanakawa, Matsuyoshi et al. 2002). In support of this idea it has been shown that *Dsc2* mRNA is upregulated in the *Dsc1* knockout mouse (M Chidgey, unpublished), although in the case of the knockout mice the increase in *Dsc2* mRNA was not translated into a significant increase in Dsc2 protein levels (Chidgey, Brakebusch et al. 2001). It should be noted that de novo expression of DSC1 and DSC3 were seen in some samples which appeared to have the normal amount of DSC2 protein product, suggesting that as simple feedback mechanism is unlikely to be the entire story.

De novo expression of the desmosomal cadherins DSC1 and DSC3 in colorectal cancer cells could have unexpected consequences. Inappropriate expression of Dsc3 in the suprabasal layers of transgenic mouse epidermis (it is normally expressed in basal cell layers) has been shown to increase β -catenin transcriptional activity (Hardman, Liu et al. 2005). Similarly, Dsg3 misexpression in suprabasal layers resulted in hyperproliferation and abnormal differentiation of the epidermis (Merritt, Berika et al. 2002). Moreover, suprabasal misexpression of Dsg2 in the epidermis of transgenic mice had profound effects - hyperproliferative skin, resistance to apoptosis and activation of cell signalling pathways (Brennan, Hu et al. 2007). The possible consequences that loss of DSC2, and gain of DSC1 and DSC3, may have on the behaviour of colonic epithelial cells in colorectal cancer remain to be determined but it is a fascinating area for future investigation. However, on a cautionary note it should be remembered that in one experiment misexpression of Dsc1 in the basal layer of the epidermis resulted in its incorporation into basal layer desmosomes without an obvious change in skin phenotype. Thus it may be that Dsc1 and Dsc3 have identical functions, at least in the basal layers of the skin (Henkler, Strom et al. 2001).

In general, the increases in mRNA expression were much greater in the desmogleins than in the desmocollins. This could have profound consequences if the large increases in desmoglein mRNAs were accompanied but similarly large increases in functional desmoglein protein.

Alterations in the relative expression of DSG1 to DSC1 have been shown to alter desmosomal adhesion (Getsios, Amargo et al. 2004). This change in stoichiometry may result in different DSC-DSG pairings, or indeed alterations in cell signalling. Desmogleins have a much greater affinity for binding to plakoglobin than desmocollins (Chitaev, Leube et al. 1996; Kowalczyk, Borgwardt et al. 1996), so changes in stoichiometry could affect intracellular signal transduction pathways (Kolligs, Kolligs et al. 2000).

6.3 Cadherin switching in cancer

The epithelial-mesenchymal transition is a biological process that takes place during embryonic development (Thiery 2003), but also in disease (Thiery 2002). EMT is characterised by loss of E-cadherin expression and gain of expression of the 'mesenchymal' cadherin N-cadherin. Activation of tyrosine kinases, expression of zinc finger binding proteins such as Snail and Slug, and activation of matrix metalloproteinase activity (Sipos and Galamb 2012) are all characteristics of EMT. In cancer EMT it has been shown to contribute to increased motility of tumour cells and increased invasive potential (Agiostratidou, Hult et al. 2007). The role of EMT has been demonstrated in the development of colonic disease. Loss of E-cadherin expression, accompanied by gain of P-cadherin expression, has been documented in radiation proctitis and early colorectal neoplasia (Hardy, Brown et al. 2002; Hardy, Tselepis et al. 2002). Induction of EMT has been implicated in progression of colorectal cancer (Gulhati, Cai et al. 2009) and the regulation of metastasis (Thiery 2002; Lee, Dedhar et al. 2006) and changes have been noted in the TGF β , Akt and wnt signalling pathways (Rychahou, Kang et al. 2008).

As a major player in epithelial adhesion, loss of desmosomal adhesion may trigger an EMT and drive cancer progression. A number of factors could result in desmosome dissolution and EMT. The transcription factor Slug, is upregulated in EMT and has been shown to cause desmosome dissociation (Savagner, Yamada et al. 1997). In addition, both Slug and Snail induce

downregulation of DSG2 in ovarian cancer cells (Kurrey, Amit et al. 2005). The epidermal growth factor receptor (EGFR) is upregulated in EMT and tyrosine phosphorylation of proteins by EGFR is a critical step in the epithelial-mesenchymal transition (Thiery 2003). Plakoglobin has been identified as a target of the EGFR pathway and tyrosine phosphorylation of desmogleins results in their reduced expression and loss of membrane localisation in keratinocytes, (Yin, Getsios et al. 2005) and simple epithelial cell lines (Miravet, Piedra et al. 2003). It is noteworthy that EGFR inhibition has also been shown to promote desmosome assembly in head and neck cancers, with increased expression of DSC2 and DSG2, and this is coincident with a change in morphology from fibroblastic to epithelial (Lorch, Klessner et al. 2004). It remains to be seen whether any of these factors are involved in the loss of expression of DSC2 that occurs in colorectal cancer, and indeed whether loss of DSC2 is able to trigger EMT and cancer. Whether DSC1 and DSC3 act as 'mesenchymal' cadherins and promote EMT remains to be determined.

6.4 Intracellular signalling

The changes in desmosomal cadherin expression observed in colorectal cancer may affect intracellular signal transduction pathways. An obvious candidate for mediating signals through this route is plakoglobin, which has well-established roles as both a structural component of desmosomes and as an agent of wnt signalling in colon cancer cells (Kolligs, Kolligs et al. 2000). It is able to behave in a similar manner to β -catenin and is targeted by APC-proteasome complex for degradation. The desmosomal cadherins are able to

bind directly to plakoglobin, with loss of the desmoglein C-terminal region resulting in reduced plakoglobin binding (Chitaev, Averbakh et al. 1998). Thus, production of a truncated desmosomal protein product or reduced expression may influence plakoglobin-mediated signals.

Although no change in *DSC2* mRNA was observed, *DSC1* and *DSC3* were upregulated. The desmocollin isoforms may vary in their plakoglobin binding potential. In addition, the de novo expression of desmoglein mRNAs were far higher than those observed for their desmocollin partners. If this was translated into a functional protein, it could be anticipated that there would be an excess of desmogleins and that some of this excess protein could be released from the membrane into the cytoplasm. Evidence is emerging that non-desmosomal DSG3 is able to engage in cell signalling in keratinocytes (Tsang, Liu et al. 2010; Tsang, Brown et al. 2012). To examine this further it would be necessary to establish whether desmoglein proteins are present in colorectal cancer samples and to determine whether they co-localise with desmocollin protein at the cell membrane or are redistributed to the cytoplasm. This could be achieved by western blotting and immunohistochemistry of the tissue samples.

6.5 Functional role of desmosomal cadherins in colorectal cancer.

Cancer is a genetically unstable disease, with multiple events and pathways, not all of which contribute to the neoplastic process. As loss of E-cadherin from adherens junctions has a profound effect on the behaviour of cultured cells and cancer, it was proposed that the desmosomal cadherins could have a similar role. The investigations of chapter 4 sought to establish a potential role for desmosomal cadherins in colorectal cancer by antisense knockdown of *DSC2* and *DSG2* mRNA. Although this strategy was successful in that some knockdown of *DSC2* and *DSG2* mRNA was achieved, the knockdown of message levels were not sufficient to manifest as a change at the protein level.

Evidence is now emerging that desmosomal cadherins play a functional role in colorectal cancer. By using a small-interfering RNA knockdown technique, Kolegraff et al were able to demonstrate a role for *DSC2* in β -catenin signalling through the P13K/Akt pathway and that this may be mediated by EGFR signalling (Kolegraff, Nava et al. 2011). This study also demonstrated that *DSC2* knockdown decreased cell proliferation and promotes invasive behaviour, supporting the idea of a tumour suppressor role. However this study did not report any changes in expression of the other desmocollins and a complementary study of *DSG2* knockdown would be very informative.

6.6 Transcriptional regulation

Little is known about the regulation of desmosomal cadherin gene expression. It is thought to take place in a coordinated fashion as the genes are clustered together on chromosome 18q. In this investigation, C/EBP β activated both the *DSC2* and *DSG2* promoters. Of the other C/EBPs, C/EBP δ had a moderate transactivating effect on the *DSC2* promoter and both C/EBPs α and δ activated the *DSG2* promoter. C/EBP α is considered to have an antiproliferative role, following the observations that it is able to mediate cell-cycle arrest (Umek, Friedman et al. 1991), is expressed in high levels in terminally differentiated cells (Birkenmeier, Gwynn et al. 1989; Cao, Umek et al. 1991) and is a potent inhibitor of proliferation when overexpressed in cultured cells (Hendricks-Taylor and Darlington 1995). In contrast to this, C/EBP β is considered to be pro-proliferative, with high levels of expression seen following partial hepatectomy and in several tumours, including colorectal cancer (Rask, Thörn et al. 2000) and breast cancer (Milde-Langosch, Löning et al. 2003). Interestingly, the C/EBPs had a much greater activating effect on the promoters of those desmocollins that are not normally expressed in colorectal cells (i.e. *DSC1* and *DSC3*) than on that of *DSC2* which is normally expressed in colorectal cells. However, it is unlikely that changes in C/EBP expression that may occur in colorectal cancer could alone explain the changes in desmosomal cadherin expression that we have observed (Chapter 3). Overexpression of, for example, C/EBP β could be responsible for the aberrant expression of *DSC3* in particular. However, one might expect that overexpression of C/EBP β to result in increased *DSC2*

mRNA and protein, neither of which were observed in colorectal cancer samples.

6.7 Future directions

The role of desmosomal cadherins in cancer is increasingly being acknowledged and the publication arising from this investigation appears to have renewed interest in their role in colorectal cancer. Of course, these experiments are preliminary and beg more questions than they answer. There are several avenues to explore. Firstly, from this study, it is not possible to say where in the adenoma-carcinoma sequence these changes take place. Further samples would have to be studied, including adenomas and cancers at all stages of the disease to elucidate the timing of these events. In addition to this, background molecular information could be collected about the tumour samples used, in order to investigate whether the observed changes in desmosomal cadherin expression can be attributed to a particular molecular pathway, namely the chromosomal instability, microsatellite instability or serrated pathway.

A second line of investigation could look at *DSG2* knockdown using a similar strategy to that used for the successful knockdown of *DSC2* (Kolegraff, Nava et al. 2011). As part of this, supporting evidence for concomitant upregulation of other desmosomal cadherins would also be sought. In addition, stable transfection experiments to over-express *DSC1*, *DSC3*, *DSG1* and *DSG3*, separately or together in colon, as well as other epithelial cell lines, would

allow the effects of de novo expression to be investigated, in particular cell adhesion and migration. Transient transfections with the Topflash plasmid could allow β -catenin activity to be investigated (Hardman, Liu et al. 2005).

A third potential line of investigation would be to investigate further the transcriptional regulation of desmosomal cadherins in colon cancer cells. Confirmation of a role for the C/EBPs, both in colon cancer and desmosomal gene regulation, is urgently required. Recent studies have shown that the transcription factors CDX1 and CDX2 are able to regulate *DSC2* activity in colon cancer cells (Funakoshi, Ezaki et al. 2008). As discussed earlier, desmosomal cadherins have been implicated in EGFR pathways and epithelial-mesenchymal transition and the transcription factor Slug causes desmosome dissociation (Savagner, Yamada et al. 1997). The effect of Slug and other EMT-regulators such as Snail and Twist on the desmosomal cadherin promoter activity would provide a further line of investigation.

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